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(57) Abstract

The present invention demonstrates the ability of IAP member proteins and caspases which associate with them to modulate granulosa cell apoptosis, an event which leads to follicular atresia. This discovery allows the development and the identification of novel methods and compounds for controlling follicular atresia. Such methods and compounds are useful in the treatment of female infertility, and in the treatment and prevention of ovarian cancer.

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MODULATORS OF OVARIAL APOPTOSIS RELATED TO IAP

Background of the Invention

In the mammalian ovary, 99.9% of oocytes and follicles are removed through the process of follicular atresia. The fate of a follicle (growth versus atresia) is determined by the actions and interactions of gonadotropins and intraovarian regulators at the cellular level, but the mechanisms regulating follicular atresia are poorly understood. *In vitro* studies have suggested that gonadotropins and intraovarian regulators are involved in the control of granulosa cell apoptosis and thus the survival of the follicle, but factors regulating apoptosis *in vivo* in early antral follicles have not been identified.

Recently it has become apparent that apoptosis is the cellular mechanism underlying follicular atresia. Apoptosis is a physiological form of cell death characterized by the degradation of cellular DNA into oligonucleosome fragments that appear as a ladder pattern when resolved by electrophoresis. Studies of the regulation of apoptosis in the ovary are therefore essential to the elucidation of the control mechanisms involved in follicular atresia.

In vitro studies of preovulatory follicles and isolated ovarian cells have shown that gonadotropins, various growth factors (*e.g.*, EGF, bFGF, and IGF-1), and cytokines (*e.g.*, IL-6 and IL-1β), can induce or suppress apoptosis in the ovary. Other *in vivo* studies have shown that preantral or immature follicles in diethylstilbestrol (DES)-treated rats undergo apoptosis after estrogen withdrawal or in response to exogenous GnRH or androgen. Follicles from DES-treated rats cultured in the absence of serum undergo apoptosis, which can be prevented by follicle-stimulating hormone (FSH), suggesting that FSH may be an important survival factor early in the process of follicle selection and development. Whether FSH is indeed a survival factor at the penultimate stage of follicular development *in vivo* remains to be determined.

Although studies have shown that atresia can occur at all stages of follicular development, the physiological significance of inducing apoptosis in undifferentiated granulosa cells in early antral or differentiated granulosa cells in preovulatory follicles is suspect, since these follicle sizes are rarely seen to undergo atresia in cycling rats. FSH stimulates the differentiation of granulosa cells and progression of follicles from the preantral to the antral stages of development. Small antral follicles beginning the process of granulosa cell differentiation are the predominant follicle type observed undergoing atresia *in vivo*, suggesting that this stage of development and differentiation is a turning point in the determination of the fate of the follicle.

Summary of the Invention

We have created a model of follicular atresia based on the withdrawal of gonadotropin with an anti-eCG antibody to study the onset and occurrence of apoptosis in eCG-primed rat ovaries. Using this approach, we have discovered that NAIP and IAP proteins are modulating factors in follicular atresia. From this discovery we can predict specific therapeutic compounds and methods of screening for therapeutic compounds. General methods and compounds relating to the IAPs are described in USSN's 08/511,485, 08/576,956, 60/017,354, 60/030,590, 08/844,693, 08/913,322, and 08/800,929, and PCT applications PCT/IB97/00721, PCT/IB97/00142, and PCT/IB96/01022, hereby incorporated by reference.

In a first aspect, the invention provides a method of decreasing apoptosis in an ovarian cell that includes administering to the ovarian cell an apoptosis-inhibiting amount of an IAP polypeptide or fragment thereof.

In a second aspect, the invention provides a method of decreasing apoptosis in an ovarian cell that includes administering a compound which increases biological activity of an IAP polypeptide. In preferred embodiments of this aspect of the

invention, the compound is nucleic acid encoding the IAP polypeptide, or is gonadotropin.

In a preferred embodiment of the first two aspects of the invention, the IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

In a third aspect, the invention provides a method of decreasing apoptosis in an ovarian cell that includes administering to the ovarian cell an apoptosis-inhibiting amount of a caspase-inhibiting caspase polypeptide fragment. Preferably, the caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

In a fourth aspect, the invention provides a method of decreasing apoptosis in an ovarian cell that includes administering to the ovarian cell a compound which decreases biological activity of a caspase polypeptide. In preferred embodiments of this aspect of the invention, the compound is a caspase polypeptide antisense nucleic acid, is an antibody which specifically binds a caspase polypeptide (*e.g.*, a caspase neutralizing antibody), or is gonadotropin. Preferably, the caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

In various embodiments of the first four aspects of the invention, the ovarian cell is in a mammal, preferably a human, which is diagnosed as having female infertility or is predisposed to developing female infertility. In another embodiment, the ovarian cell is a granulosa cell.

In a fifth aspect, the invention provides a method of inducing apoptosis in an ovarian cell that includes administering to the ovarian cell an apoptosis inducing amount of an IAP-inhibiting IAP polypeptide fragment. In a preferred embodiment of this aspect of the invention, the IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

In a sixth aspect, the invention provides a method of inducing apoptosis in an ovarian cell that includes administering a compound which decreases biological activity of an IAP polypeptide. In preferred embodiments of this aspect of the invention, the compound is an IAP polypeptide antisense nucleic acid or an antibody

which specifically binds to an IAP member protein (*e.g.*, an IAP neutralizing antibody). In another preferred embodiment of this aspect of the invention, the IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

In a seventh aspect, the invention provides a method of inducing apoptosis in an ovarian cell that includes administering to the ovarian cell an apoptosis inducing amount of a caspase polypeptide or fragment thereof. Preferably, the caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

In an eighth aspect, the invention features a method of inducing apoptosis in an ovarian cell that includes administering a compound which increases biological activity of a caspase polypeptide. Preferably, the caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

In various embodiments of the fifth, sixth, seventh, and eighth aspects of the invention, the ovarian cell is in a mammal, preferably a human, which is diagnosed as having ovarian granulosa cell hyperplasia.. In another embodiment, the ovarian cell is a granulosa cell.

In a ninth aspect, the invention provides a method of identifying a compound that modulates apoptosis in an ovarian cell that includes: (a) providing a cell expressing an IAP polypeptide; and (b) contacting the cell with a candidate compound and monitoring the expression of the IAP polypeptide, where an alteration in the level of expression of the IAP polypeptide indicates the presence of a compound that modulates apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In a tenth aspect, the invention provides a method of identifying a compound that modulates apoptosis in an ovarian cell that includes: (a) providing a cell expressing a functional IAP polypeptide; and (b) contacting the cell with a candidate compound and monitoring the biological activity of the IAP polypeptide, where an alteration in the level of biological activity of the IAP polypeptide indicates the

presence of a compound that modulates apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In an eleventh aspect, the invention provides a method of identifying a compound that modulates apoptosis in an ovarian cell that includes: (a) providing a cell including a reporter gene operably linked to a promoter from a gene encoding an IAP polypeptide; (b) contacting the cell with a candidate compound; and (c) measuring expression of the reporter gene, where an alteration in the expression in response to the candidate compound indicates the presence of a compound that modulates apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In various embodiments of the ninth, tenth, and eleventh aspects of the invention, an alteration that is an increase indicates the compound decreases apoptosis and an alteration that is a decrease indicates the compound increases apoptosis. In a preferred embodiment, the IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP. In another preferred embodiment of these aspects of the invention, the ovarian cell is a granulosa cell.

In an twelfth aspect, the invention provides a method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell that includes: (a) providing a cell expressing a caspase polypeptide; and (b) contacting the cell with a candidate compound and monitoring the expression of the caspase polypeptide, where an alteration in the level of expression of the caspase polypeptide indicates the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In a thirteenth aspect, the invention provides a method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell that includes: (a) providing a cell expressing a functional caspase polypeptide; and (b) contacting the cell with a candidate compound and monitoring the biological activity of the IAP polypeptide, where an alteration in the level of biological activity of the polypeptide

indicates the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In a fourteenth aspect, the invention features a method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell that includes: (a) providing a cell including a reporter gene operably linked to a promoter from a gene encoding a caspase polypeptide; (b) contacting the cell with a candidate compound; and (c) measuring expression of the reporter gene, where an alteration in the expression in response to the candidate compound indicates the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In a fifteenth aspect, the invention features a method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell that includes: (a) providing a caspase polypeptide, the polypeptide isolated from a cellular extract; (b) contacting the caspase polypeptide with a candidate compound; and (c) measuring ability of the caspase polypeptide to cleave a caspase substrate, where an alteration in the ability in response to the candidate compound indicates the presence of compound that modulates IAP-associated apoptosis in an ovarian cell. In one embodiment of this aspect of the invention, the cell is an ovarian cell.

In various embodiments of the twelfth, thirteenth, fourteenth, and fifteenth aspects of the invention, an alteration that is an increase indicates the compound increases apoptosis and an alteration that is a decrease indicates the compound decreases apoptosis. In a preferred embodiment, the caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7. In another preferred embodiment of these aspects of the invention, the ovarian cell is a granulosa cell.

By "ovarian cell" is meant a cell from the ovarian tissue. Ovarian cells include, without limitation, oocytes, follicular cells, granulosa cells, theca cells, ovarian fibroblasts, and ovarian neuronal cells.

By "modulating apoptosis" or "altering apoptosis" is meant increasing or decreasing the number of cells that would otherwise undergo apoptosis in a given cell population. Preferably, the cell population is selected from a group of ovarian cells including, without limitation, oocytes, follicular cells, granulosa cells, theca cells, ovarian fibroblasts, or ovarian neuronal cells. It will be appreciated that the degree of modulation provided by an IAP member protein or a modulating compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change in the level of apoptosis which identifies a reagent or compound which modulates apoptosis.

By "inhibiting apoptosis" is meant any decrease in the number of cells which undergo apoptosis relative to an untreated control. Preferably, the decrease is at least 25%, more preferably the decrease is 50%, and most preferably the decrease is at least one-fold.

By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene, preferably, an IAP-encoding gene (*e.g.*, HIAP-1, HIAP-2, or XIAP) or a caspase-encoding gene (*e.g.*, caspase-3 or caspase 7). An antisense nucleic acid is capable of preferentially lowering the activity of an IAP or caspase polypeptide encoded by an IAP or caspase gene, respectively.

By "caspase" or "caspase family member" is meant a meant a polypeptide, or fragment thereof, which is derived from a member of the caspase family of proteins. Caspase family member proteins include, without limitation, caspase-1 (also known as ICE, for interleukin-1β-converting enzyme; Cerretti *et al.*, Science 256 :97-100, 1992; Alnemri *et al.*, J. Biol. Chem. 270: 4312-4317, 1995), caspase-3 (also known as CPP32, apopain, and YAMA; Tewari et al., Cell 81: 801-809, 1995), caspase-7 (also

known as LAP3 and Mch3; Duan *et al.*, J. Biol. Chem. 271: 1621-1625, 1996), and caspase-8 (also known as FLICE, Mach, and Mch5; Muzio *et al.*, Cell 85: 817-827, 1996). Preferably, such a polypeptide has an amino acid sequence which is at least 45%, preferably 60%, and most preferably 85% or even 95% identical to at least one of the amino acid sequences of the caspase-1, caspase-3, caspase-7, or caspase-8 polypeptides described in the references provided herein. The biological activities of a full length caspase family members includes a protease activity which may be measured by any standard biochemical assay, such as the caspase activity assay described in Deveraux *et al.*, Nature 388: 300-304, 1997.

By "IAP" or "an IAP polypeptide" is meant a polypeptide, or fragment thereof, which is derived from a member of the IAP family of proteins. A member of the IAP family of proteins is an amino acid sequence which has identity to baculovirus inhibitors of apoptosis. For example, NAIP, HIAP1, HIAP2 and XIAP are specifically included as members of the IAP family of proteins (see USSN's 08/511,485 filed August 4, 1995; 08/576,956 filed December 22, 1995; 60/017,354 filed April 26, 1996; 60/030,590 filed November 14, 1996; 08/844,693 filed April 25, 1997; 08/913,322 filed September 12, 1997; 08/800,929 filed February 13, 1997; PCT/IB97/00721 filed April 25, 1997; PCT/IB97/00142 filed January 17, 1997; and PCT/IB96/01022 filed August 5, 1996). Preferably, such a polypeptide has an amino acid sequence which is at least 45%, preferably 60%, and most preferably 85% or even 95% identical to at least one of the amino acid sequences of the NAIP, HIAP1, HIAP2, or XIAP polypeptides described in the references provided herein.

By "IAP-associated apoptosis" is meant apoptosis (*i.e.*, programmed cell death) that is affected by the increase or decrease in the expression or activity of a member of the IAP family of proteins.

By "polypeptide" is meant any chain of more than two amino acids, regardless of post-translational modification such as glycosylation or phosphorylation.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, *e.g.*, an antibody specific for an IAP family member protein or an antibody specific for a caspase family member protein. A purified antibody may be obtained, for example, by affinity chromatography using recombinantly-produced protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody that recognizes and binds a protein but that does not substantially recognize and bind other molecules in a sample, *e.g.*, a biological sample, that naturally includes protein. One preferred antibody binds to an IAP polypeptide (*e.g.*, XIAP). Another preferred antibody binds to a caspase polypeptide (*e.g.*, caspase-3).

By "neutralizing antibody" is meant an antibody that interferes with any of the biological activities of a polypeptide, particularly the ability of an IAP family member protein or a caspase family member protein to participate in apoptosis. The neutralizing antibody may reduce the ability of an IAP polypeptide or a caspase polypeptide to participate in apoptosis by, preferably 50%, more preferably by 70%, and most preferably by 90% or more. Any standard assay of apoptosis, including those described herein, may be used to assess potentially neutralizing antibodies.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (*i.e.*, facilitates the production of, *e.g.*, a polypeptide, a recombinant protein or a RNA molecule).

By "reporter gene" is meant any gene which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (*e.g.*, green fluorescent protein), enzymatic activity (*e.g.*, luciferase or chloramphenicol acetyl transferase), toxicity

(*e.g.*, ricin), or an ability to be specifically bound by a second molecule (*e.g.*, biotin or a detectably labelled antibody).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequences.

By "hyperplasia" is meant any abnormal proliferation of cells. One example of hyperplasia is cancer. A hyperplasia specifically included in aspects of the invention is ovarian granulosa cell hyperplasia.

By "predisposed to developing female infertility" is meant any female mammal having difficulty conceiving an offspring over a period of a year or more. Specifically, this definition in includes an individual who has undergone chemotherapy (*e.g.*, an individual who has been treated with adriamycin, also know as doxorubicin).

Brief Description of the Drawings

Figs. 1A and 1B are photomicrographs of histological sections of rat ovaries. Ovaries were removed, processed, and stained with hematoxylin/phloxine/saffron 24 hours following no treatment (Fig. 1A) or anti-eCG antibody-treatment (Fig. 1B). Arrows indicate examples of atretic follicles. Arrowhead indicates an oocyte within the discus proligerous of a healthy follicle.

Figs. 2A, 2B, and 2C are photomicrographs of viable staining for cell death in rat ovaries. Control (Fig. 2A at X40 and Fig. 2B at X160) and anti-eCG antibody-treated (Fig. 2C at X160) rat ovaries were stained with acridine orange (5 µg/ml; 5 minutes), and visualized with fluorescence microscopy using an FITC filter. Nuclear material in dead cells stain brightly compared to background.

Figs. 3A, 3B, 3C, 3D, 3E, and 3F are photomicrographs identifying cell death in rat ovarian follicles. Fluorescent TUNEL staining of anti-eCG antibody treated (Figs. 3A, 3B, 3C, and 3D) and control (Fig. 3E) rat ovaries removed 24 hours after treatment is shown. A higher magnification (X400) of an atretic follicle from an antibody treated rat (Fig. 3B, TUNEL; Fig. 3C, phase contrast) demonstrated the occurrence of cell death in granulosa (labelled G), but not theca (labelled T) cells. Granulosa cell death was prevalent in small- and medium-sized follicles of antibody-treated rats (Figs. 3A and 3B), and was less frequently observed in large follicles of antibody-treated (Fig. 3D) or control (Fig. 3E) rat ovaries. Note the occurrence of cell death in the ovarian surface epithelial cells in Figs. 3D and 3E. A positive control (Fig. 3F) for TUNEL, produced by treating the ovarian section with DNase I, showed fluorescence in all cell nuclei. Arrowheads indicate dead cells. Arrows indicate follicles containing dead cells.

Figs. 4A and 4B are photomicrographs (X400) of Hoechst-stained granulosa cells. Control (Fig. 4A) and anti-eCG antibody-treated (Fig. 4B) rat ovaries were punctured to collect granulosa cells, which were stained with Hoechst 33248 (0.1 µg/ml) to visualize nuclear DNA, and observed with fluorescence microscopy. Arrowheads indicate apoptotic nuclei.

Figs. 5A, 5B, and 5C are bar graphs (Figs. 5A and 5B) and an electrophoresed agarose gel (Fig. 5C) showing physical (Fig. 5A), biochemical (Fig. 5B), and nuclear

(Fig. 5C) indications of atresia and apoptosis in control and anti-eCG antibody-treated rat ovaries. Control (con) and antibody (Ab)-treated ovaries were removed 24 hours after treatment and examined for ovarian weight (Fig. 5A), Hoechst-stained apoptotic cell number (Fig. 5B), and apoptosis by DNA laddering (Fig. 5C). Significant difference between Ab and con was found in Figs. 5A and 5B ($p<0.05$).

Fig. 6 is a bar graph showing the induction of DNA ladders in granulosa cells by anti-eCG antibody. Granulosa cell DNA from control (con, open bars) and antibody- (Ab, closed bars) treated rats was 3'-end labelled with [^{32}P]dCTP and analyzed by agarose gel electrophoresis. Densitometric counting of low molecular weight DNA is shown, where * indicates $p<0.05$ and ** indicates $p<0.01$ significant difference from con at 1 hour.

Fig. 7 is a series of photographs showing the co-localization of normal and apoptotic cells and XIAP and HIAP-2 in ovarian sections from rats undergoing gonadotropin withdrawal *in vivo*, demonstrating that XIAP and HIAP-2 were highly expresed in normal (healthy follicles; right column of three photographs) granulosa cells, but not in apoptotic (atretic follicles; left column of three photographs) granulosa cells.

Figs. 8A, 8B, 8C, 8D, 8E, 8F, 8G, 8H, 8I, 8J, 8K, 8L, 8M, 8N, 8O, and 8P are photographs showing immunolocalization of PCNA and IAPs in rat ovarian follicles at different stages of development. Ovaries from immature rats treated with either DES (1 mg/day, 3 consecutive days; for preantral and early antral follicles) or eCG (15 IU; for mid to late antral follicles) were fixed and adjacent paraffin sections were cut. Cell death was detected by TUNEL. PCNA, HIAP-2 and XIAP proteins were localized with corresponding specific antibodies by the ABC (avidin-biotin complex) method. Figs. 8A, 8B, 8C, 8D; 8E, 8F, 8G, 8H; 8I, 8J, 8K, 8L; and 8M, 8N, 8O, 8P

show positivities and immunoreactivities for TUNEL, PCNA, HIAP-2, and XIAP, respectively. With the exception of follicle "A" in Fig. 8D (oocyte not shown in section, which is at the early antral stage), Figs. 8A, 8E, 8I, 8M; 8B, 8F, 8J, 8N; 8C, 8G, 8K, 8O and 8D, 8H, 8L, 8P indicate preantral, early antral, and mid antral atretic ("A") or healthy ("H") follicles, respectively. The designations are as follows: O, oocyte; GC, granulosa cells; TC, theca cells; IC, interstitial cells; and SE, surface epithelial cells.

Figs. 9A, 9B, 9C, 9D, 9E, and 9F are photographs of slide sections showing *in situ* detection of apoptosis and immunolocalization of HIAP-2 and XIAP in medium sized antral follicles from eCG (15 IU)-treated immature rats. Using adjacent paraffin sections, HIAP-2 (Figs. 9C and 9D) and XIAP (Figs. 9E and 9F) proteins were localized with corresponding specific antibodies by ABC (avidin-biotin complex) method. Arrow heads, nuclear immuno-reactivity; arrows, cytoplasmic immuno-reactivity.

Fig. 10 is a photograph of a Western blot analysis showing ovarian follicular development induced by injection of eCG, and suppressed granulosa cell apoptosis which was associated with increased HIAP-2 protein expression. Follicular stage is as follows: E, early stage (preantral and early antral follicles); M, mid stage (small to medium-sized antral follicles); and L, late stage (preovulatory follicles).

Figs. 11A, 11B, 11C, 11D, 11E, and 11F are photographs of Western blots (Figs. 11A and 11C) and an autoradiogram of DNA fragmentation (Fig. 11E) densitometrically analyzed and represented as bar graphs (Fig. 11B, 11D, and 11F) showing the expression of IAPs (Figs. 11A-11D) and apoptotic DNA degradation (Figs. 11E and 11F) in granulosa cells during follicular development induced by DES (1 mg/day, 3 consecutive days); eCG (15 IU); and eCG (15 IU) plus hCG(15 IU; 48

hours post-eCG) treatment *in vivo*. IAPs in granulosa cell protein extracts (100 µg/lane) were analyzed by Western blot, and extracted DNA (500 ng) were labelled with radioisotope (^{32}P-dCTP), and resolved on agarose gel. Fig. 11A and 11C are representative immunoblots of HIAP-2 and XIAP proteins, respectively. Fig. 11E shows a representative autoradiogram of DNA fragmentation analysis. Changes in IAPs and low molecular weight DNA contents as analyzed densitometrically (Image Analysis Systems from Bio-Rad Laboratories) are shown in Figs. 11B, 11D, and 11F, respectively. Data represent ± SEM of four experiments. The designation, *, indicates $P<0.05$ (compared with DES group). The designation, #, indicates $P<0.001$ (compared with eCG plus hCG group).

Figs. 12A, 12B, 12C, 12D, 12E, and 12F are photographs of Western blots (Figs. 12A and 12C) and an autoradiogram of DNA fragmentation (Fig. 12E) densitometrically analyzed and represented as bar graphs (Fig. 12B, 12D, and 12F) showing the expression of IAPs (Figs. 12A-12D) and apoptotic DNA degradation (Figs. 12E and 12F) in rat granulosa cell during follicular atresia induced by eCG withdrawal with eCG-antibody treatment *in vivo*. Figs. 12A and 12C are representative immunoblots of HIAP-2 and XIAP proteins, respectively. Fig. 12E shows a representative autoradiogram of DNA fragmentation analysis. Changes in IAPs and low molecular weight DNA contents as analyzed densitometrically are shown in Figs. 12B, 12D, and 12F, respectively. Data represent ± SEM of four experiments. The designation, *, indicates $P<0.05$ (compared with 24 hours control). The designation, +, indicates $P<0.05$ (compared with 1 hour control). The designation, #, indicates $P<0.001$ (compared with 1 hour control). Con represents control groups treated with pre-immune rabbit serum, while Ab indicates anti-eCG antibody groups.

Fig. 13A is a Western blot analysis showing the suppression of HIAP-2 protein content in anti-eCG antibody-treated (Ab) granulosa cells following gonadotropin withdrawal.

Fig. 13B is a Western blot analysis showing the suppression of XIAP protein content in anti-eCG antibody-treated (Ab) granuloma cells following gonadotropin withdrawal.

Description of the Preferred Embodiments

Apoptosis is the cellular process that underlies follicular atresia, which accounts for the loss of most follicles from the adult mammalian ovary. To fully elucidate the role and regulation of the apoptosis process in the ovary, models for the *in vivo* induction of ovarian apoptosis are necessary. We have developed an *in vivo* model that is ideal for the examination of the earliest events of apoptosis at the critical penultimate stage of follicle development. Using our model, we have discovered that HIAP-2 and XIAP, two proteins from the family of proteins known as the IAPs (Inhibitor of Apoptosis Proteins), are involved in the suppression of granulosa cell apoptosis by gonadotropin in small to medium sized antral follicles and play an important role in determining the fate of the cells, and eventual follicle destiny (atresia or ovulation). Use of our finding, that a decrease in the cellular protein content of HIAP-2 or XIAP correlates with an increase in follicular atresia, allows the generation of methods and compounds for controlling follicular atresia. Such methods and compounds are useful for treating female infertility (including polycystic ovarian diseases), and are also useful in the treatment and prevention of ovarian cancer.

Identification of Compounds that Modulate Follicular Atresia

In the model system described herein, follicular maturation and growth is promoted by the addition of either chorionic gonadotropin (equine or human) or

diethylstilbestrol (DES). Withdrawal of chorionic gonadotropin, following treatment with this hormone, results in granulosa cell apoptosis and follicular atresia. Our findings have shown that cellular protein content of a member of the IAP family of proteins directly affects the fate of the follicle, where a reduced level of an IAP member protein leads to follicular atresia.

Given our finding that an increase in protein expression of IAP family member proteins (*e.g.*, HIAP-2 and XIAP) leads to the prevention of granulosa cell apoptosis and, consequently, the prevention of follicular atresia, compounds, such as drugs or other therapeutics that affect IAP protein expression are useful in modulating follicular atresia. A compound which increases IAP expression, and thereby prevents follicular atresia, is useful in the treatment of female infertility, especially in patients who are incapable of responding to gonadotropin.

Similarly, since XIAP has recently been found to inhibit activity of at least two members of the caspase family of cell death inducers, caspase-3 and caspase-7 (Deveraux *et al.*, Nature 388: 300-304, 1997), compounds, such as drugs or other therapeutics that affect the protein expression of a caspase family member (*e.g.*, capsase-3 or caspase-7) are useful in modulating follicular atresia. A compound which decreases caspase expression, and thereby prevents follicular atresia, is useful in the treatment of female infertility.

IAP member protein-encoding cDNAs may be used to facilitate the identification of compounds that increase or decrease expression of one or more specific IAP member proteins. In one approach, candidate compounds are added, in varying concentrations, to the culture medium of cells expressing mRNA encoding an IAP member protein. The mRNA expression of the IAP member protein is then measured, for example, by Northern blot analysis (as described in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994) using the IAP member protein-encoding DNA, cDNA, or RNA fragment, as a hybridization probe. The level of the IAP expression in the presence of the candidate

compound is compared to the level of expression in the absence of the candidate compound, all other factors (*e.g.*, cell type and culture conditions) being equal. Similar assays may be employed using caspase family member-encoding cDNAs.

The effect of candidate compounds on IAP family member protein-mediated apoptosis or on a caspase family member protein may, instead, be measured at the level of translation by using standard protein detection techniques, such as Western blotting or immunoprecipitation with an antibody specific toward the IAP member protein of interest (*e.g.*, an XIAP-specific antibody) or the caspase family member protein of interest (*e.g.*, an caspase-3 specific antibody), respectively.

In an approach to detecting compounds which regulate IAP family members at the level of transcription, candidate compounds may be tested for an ability to regulate a reporter gene whose expression is directed by the promoter of the gene encoding the specific IAP family member. For example, a cell unlikely to undergo apoptosis may be transfected with a expression plasmid comprising a luciferase reporter gene operably linked to the IAP gene promoter. Candidate compounds may then be added, in varying concentrations, to the culture medium of the cells. Luciferase expression levels may then be measured by subjecting the compound-treated transfected cells to standard luciferase assays known in the art, such as the luciferase assay system kit used herein that is commercially available from Promega, and rapidly assessing the level of luciferase activity on a luminometer. The level of luciferase expression in the presence of the candidate compound is compared to the level of luciferase expression in the absence of the candidate compound, all other factors (*e.g.*, cell type and culture conditions) being equal. Similar assays may be employed using promoters from a gene encoding a caspase family member (*e.g.*, the caspase-3 gene).

Compounds that modulate the level of expression of IAP member proteins or caspase family member proteins may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel *et al.*, *supra*). In an assay of a mixture of compounds, protein

expression is tested against progressively smaller subsets of the compound pool (*e.g.*, produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate the expression of the protein of interest (*e.g.*, XIAP or caspase-3).

Compounds may also be screened for their ability to modulate IAP apoptosis-inhibiting activity. In this approach, the degree of apoptosis in the presence of a candidate compound is compared to the degree of apoptosis in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful modulator compounds are isolated in a step-wise fashion. Apoptosis activity may be measured by any standard assay, for example, those described herein.

Compounds or molecules that function as modulators of IAP-mediated cell death inhibition may include peptide and non-peptide molecules such as those present in cell extracts, mammalian serum, or growth medium in which mammalian cells have been cultured.

A molecule that promotes an increase in IAP expression or activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to increase cellular levels of IAP member protein and thereby exploit the ability of these polypeptides to prevent apoptosis. Such molecules are useful therapeutics in treating female infertility. Likewise, molecules found to promote a decrease in caspase expression or activity is considered useful in the invention and may be used as a therapeutic to treat female infertility. It will be understood that all therapeutic molecules of the invention may be used in combination to treat the same condition. For example, a molecule which increases XIAP expression may be used therapeutically in combination with a molecule which decreases caspase-3 expression to treat female infertility.

A molecule that promotes a decrease in IAP expression or activity is also considered useful in the invention; such a molecule may be used, for example, as a

therapeutic to decrease cellular levels of IAP member protein in the treatment and prevention of ovarian hyperplasia (*e.g.*, ovarian cancer). Likewise, molecules found to promote an increase in caspase expression or activity is considered useful in the invention and may be used as a therapeutic to treat ovarian hyperplasia. It will be understood that all therapeutic molecules of the invention may be used in combination to treat the same condition. For example, a molecule which decreases HIAP-2 expression may be used therapeutically in combination with a molecule which increases caspase-3 expression to treat ovarian cancer.

Molecules that are found, by the methods described above, to effectively modulate IAP member protein or capase family member protein expression or polypeptide activity may be tested further in animal models, such as the rat model described herein. If they continue to function successfully in an *in vivo* setting, they may be used as therapeutics to either inhibit or enhance apoptosis, as appropriate.

Test Compounds

In general, novel drugs for prevention or treatment of ovarian cell death which functions by targeting biological activity of a member of the IAP or caspase families of protein are identified from large libraries of both natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (*e.g.*, semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-,

peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for neurodegenerative disorders should be employed whenever possible.

When a crude extract is found to prevent or delay ovarian cell death, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having ovarian cell death-preventative or -palliative activities. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using a mammalian ovarian cell death model.

Below are examples of high-throughput systems useful for evaluating the efficacy of a molecule or compound in treating, preventing, or enhancing an ovarian cell death-associated condition.

Therapies

Therapies may be designed to circumvent or overcome an IAP member protein genetic defect or relatively inadequate IAP member protein expression, and thus modulate and possibly inhibit apoptosis. In considering various therapies, it is understood that such therapies may be targeted at specific tissues, for example, the ovaries. Apoptosis-inhibiting IAP member protein reagents may include, without limitation, full length or fragment IAP member polypeptides, IAP member protein-encoding mRNA, or any compound which increases IAP apoptosis-inhibiting activity. Conversely, therapies to inhibit IAP gene expression are useful in promoting apoptosis in cancerous cells.

Likewise, therapies may be designed to overcome a caspase family member protein genetic defect or surplus caspase family member protein expression, and thus inhibit apoptosis. Such apoptosis-inhibiting therapies may be targeted at the ovaries, and may include, without limitation, full length or fragment caspase family member polypeptides, caspase family member protein-encoding mRNA, or any compound which decreases caspase apoptosis-inducing activity. Conversely, therapies to induce caspase family member gene expression are useful in promoting apoptosis in cancerous cells.

a) Protein Therapy

Treatment or prevention of inappropriate or undesired apoptosis can be accomplished by delivering surplus IAP member proteins to the appropriate cells. It is also be possible to modify the pathophysiologic pathway (*e.g.*, a signal transduction pathway) in which the protein participates in order to correct the physiological defect.

To add protein to cells which do not express sufficient amounts of one or more IAP member proteins, it is necessary to obtain large amounts of pure IAP member proteins from cultured cell systems which can express the protein. Delivery of the protein to the affected tissues (*e.g.*, ovarian tissues) can then be accomplished using appropriate packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to the afflicted individual to act as IAP member protein agonists and in this manner produce a desired physiological effect. Methods for finding such molecules are provided herein.

Treatment or prevention of inappropriate or undesired apoptosis can also be accomplished by delivering to the appropriate cells reagents which reduce the level of capase family member proteins (*e.g.*, a capase family member specific neutralizing antibody, antisense caspase family member nucleic acid). Alternatively, apoptosis can be inhibited by introducing into affected cells a dominant negative caspase family member protein.

b) Gene Therapy

Gene therapy is another potential therapeutic approach in which normal copies of the IAP member protein-encoding genes or mRNA are introduced into selected tissues to successfully encode normal and abundant protein in affected cell types (*e.g.*, ovarian granulosa cells). The nucleic acids must be delivered to those cells in a form in which it can be taken up and encode for sufficient protein to provide effective function. For example, copies of mRNA encoding the desired IAP member protein may be produced in large amounts in vitro. For example, COS or HeLa cells may be transfected with a plasmid encoding the desired IAP member protein (*e.g.*, HIAP-2). mRNA encoding the desired IAP member protein may then be isolated from the cells during mRNA preparation from the cells (see techniques in Ausubel *et al.*, *supra*), and then size fractionated. The size of mRNAs corresponding to the size of mRNA encoding the desired IAP member protein may then be purified and delivered to the ovaries using appropriate packaging or administrating systems.

Using this approach, copies of caspase family member antisense nucleic acids are introduced into selected tissues to successfully produce antisense nucleic acids in affected cell types (*e.g.*, ovarian granulosa cells). Likewise, copies of genes or mRNA encoding dominant-negative mutants of caspase proteins may be introduced into affected cell types.

The caspase nucleic acids and proteins may be delivered as described above for the IAP reagents.

For any of the methods of application described above, the therapeutic proteins or nucleic acids are preferably applied to the site of the desired apoptosis-inhibiting event (for example, by injection). However, it may also be applied to tissue in the vicinity of the desired apoptosis event or to a blood vessel supplying the cells (*e.g.*, ovaries) desired to undergo prevention of apoptosis.

Administration of Caspase Member or IAP Member Polypeptides, Genes, or Modulators of Caspase Member or IAP Member Protein Synthesis or Function

A caspase member or IAP member protein, gene, or modulator may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer caspase member or IAP member proteins, IAP member protein-inducing compounds (*e.g.*, IAP member protein-encoding nucleic acid), or caspase family member-inhibiting compounds (*e.g.*, caspase family member-specific neutralizing antibody) to a patient suffering from a condition that is caused by excessive apoptosis (*e.g.*, infertility). Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracapsular, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the

form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for caspase family member protein or IAP member protein modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a caspase family member or IAP member protein, gene, or modulatory compound may be combined with more traditional therapies for the disease such as hormone therapy for infertility, or radio- and chemo-therapy for cancer.

Preventative Anti-Apoptotic Therapy

In a patient diagnosed with female infertility, any of the above therapies may be administered before the occurrence of the condition phenotype. For example, the therapies may be provided to a patient who has female infertility, but does not yet show a diminished oocyte cell count. In particular, compounds shown to increase IAP member protein expression or biological activity or decrease caspase family member

protein expression or biological activity may be administered by any standard dosage and route of administration (see above).

The methods of the instant invention may be used to reduce or diagnose the disorders described herein in any mammal, for example, humans, domestic pets, or livestock.

The following examples are meant to illustrate, not limit, the invention.

I. MODEL SYSTEM

MATERIALS AND METHODS

I. Materials

Ethanol, 10% neutral buffered foralin, xylene, paraffin, acetic acid, EDTA, and $MgCl_2$ were from BDH (Toronto, ON). Ethidium bromide, agarose, Hoescht 33248, acridine orange, Tris, PBS, protease K, bovine pancreatic DNase I, and equine chorionic gonadotropin (eCG) were from Sigma (ST. Louis, MO). Klenow enzyme and [^{32}P]dCTP were from Amersham (Arlington Heights, IL). Medium M199 and normal rabbit serum were from Gibco/BRL (Burlington, ON).

II. Animal Treatment Groups

Immature female Sprague-Dawley rats at 23 days of age were injected with eCG (15 IU, s.c.) and injected 24 hours later with normal rabbit serum (1:10 in saline; i.p) or 100 µL anti-eCG antibody (1:10 in saline; i.p.). At the indicated time after serum or antibody injection, ovaries were excised, cleared of adhering fat, weighed, and either fixed in 10% neutral buffered formalin for histology or placed in medium M199 for viable staining and granulosa cell collection.

III. Histology and Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick Endl-Labelling (TUNEL)

Ovaries fixed in 10% neutral buffered formalin were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin, sectioned (5 μm), and mounted on charged slides. Sections were then cleared in xylene and rehydrated through a graded series of ethanol to PBS. For routine histology, slides were stained with hematoxylin/phloxine/saffron (H&E), and observed and photographed by light microscopy. To identify cell death using the *in situ* cell death detection kit (Boehringer-Mannheim), slides were treated (20 minutes, 37°C) with protease K (10 μg/ml in 20 mM Tris +2 mM $CaCl_2$, pH 7.5), washed three times in PBS, and then incubated (60 minutes, 37°C) with terminal transferase in the presence of FITC-conjugated dUTP according to the manufacturers instructions (Boehringer-Mannheim). The stained sections were then photographed on a Zeiss microscope equipped with epifluorescent optics. As a positive control for *in situ* detection, slides were preincubated (20 minutes, 37°C) with bovine pancreatic DNase I (0.2 μg/ml in 20 mM Tris + 2 mM $CaCl_2$ + 5 mM $MgCl_2$, pH 7.5) before the terminal transferase reaction.

IV. Identification of Cell Death

Viable staining: Ovaries were stained in acridine orange (5 μg/ml in M199) for 5 minutes at 37°C, washed in M199 (3 times, 3 minutes each), and photographed with a Zeiss microscope using FITC optics (Delic *et al.*, Exp. Cell. Res. 194: 147-153, 1991).

Nuclear staining: Granulosa cells were collected by follicle puncture as previously described (Farooki, R., Endocrinology 110: 1061-1063, 1982), fixed (4% formalin in PBS; 10 minutes, room temperature), washed in PBS, resuspended in Hoescht 33248 staining solution (0.1 μg Hoescht 33248/ml PBS, 10 minutes), washed again, and spotted onto slides for microscopy. Nuclear staining was observed and

photographed using a Zeiss fluorescent microscope equipped with an FITC filter. Apoptotic cells were identified by typical nuclear morphology, and counted using randomly selected fields and numbered photographic slides to avoid bias during counting.

Quantification of DNA ladders: Granulosa cells were collected by follicular puncture, pelleted, snap frozen, and stored at -70°C until analysis. Cells were resuspended in 200 µL PBS, and DNA extracted using the Qiagen Blood Amp kit according to manufacturers instructions (Qiagen Inc., Chatsworth, CA). DNA was quantified by ethidium bromide fluorescence using standard techniques. Briefly, 5 µL of sample was spotted onto a UV transilluminator covered with saran wrap, mixed with 5 µL ethidium bromide stain (0.2 µg/ml in 20 nM Tris), and quantified by comparison of the fluorescence intensity of DNA standards using the Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, CA). DNA (0.5 µg) was end labelled as described (Rosl, F., Nucl. Acid Res. 20: 5243, 1992) by incubating (20 minutes, room temperature) with Klenow enzyme (2 U in 10 mM Tris + 5 mM $MgCl_2$) and 0.1 µCi [^{32}P]dCTP. Unincorporated nucleotides were removed with the Qiagen Nucleotide Removal kit, and samples were resolved by Tris-acetate-EDTA agarose (1.8%) gel electrophoresis. The gel was then dried (2 hours, no heat) and exposed to a Bio-Rad phosphorimager screen to densitometrically quantify low molecular weight DNA (<15 Kbp), and subsequently exposed to X-ray film at -80°C.

RESULTS

I. Induction of Follicular Atresia by Anti-eCG Antibody

Gonadotropin is an important survival factor for the developing follicles to escape atresia and reach the preovulatory follicle stage (Byskov, A.G., " Follicular atresia," The Vertebrate Ovary, Comparative Biology and Evolution, ed. R. E. Jones, Plenum, New York, pp 533-562, 1978, Billig *et al.*, Endocrinology 134: 245-252,

1994; Chun *et al.*, Endocrinology 135:1845-1853, 1994, Hirshfield and Midgley, Biol. Reprod 19: 606-611, 1978; Schwartz N.B., Biol. Reprod. 10: 236-276, 1974). Rats were injected with equine chorionic gonadotrophoin (eCG), and 24 hour later with anti-eCG antibody or normal rabbit serum (control), and ovaries were removed 24 hours after treatment for histological examination. Figs. 1A and 1B show a typical result of the antibody treatment on gross ovarian morphology. Control ovaries (Fig. 1A) contained numerous large antral follicles at the preovulatory stage. Several layers of granulosa and theca cells were evident, and healthy oocytes were often found within the discus proligerous. In contrast, ovaries from antibody-treated animals (Fig. 1B) contained numerous small to medium sized follicles and some larger follicles. Granulosa cell layers were uneven and occasionally detached from the basal lamina, and some loose granulosa cells could be seen within the antral cavity. Ovarian size in the antibody-treated group was notably smaller than that of the controls, and was reflected in a significant difference in ovarian weight (see Fig. 5A).

II. Localization of Cell Death in the Ovary

Figs. 2A, 2B, and 2C show whole ovaries removed 24 hours after treatments, stained with acridine orange (Delic *et al.*, *supra*), and viewed by fluorescent microscopy to visualize dying cells, which are identified by brightly staining DNA. Cell death was apparent in the granulosa but not the theca cell layers, of small to medium sized antral follicles of anti-eCG treated rat ovaries (Fig. 2C). Most large preovulatory follicles did not display any cell death. In those few large follicles where cell death was evident, only a small number of dead granulosa cells could be seen. Similarly, some dead cells were seen in control ovaries, although most of the follicles, regardless of size, did not contain large numbers of brightly stained dead cells. These observations were confirmed with TUNEL labelling of histological sections. Figs. 3A, 3B, 3C, 3D, 3E, and 3F show a typical result comparing the incidence of DNA fragmentation in follicles from control and antibody-treated rats. Granulosa cells in

small to medium sized antral follicles of the antibody-treated group were 3'-end labelled with FITC-conjugated dUTP when incubated with terminal transferase, indicative of DNA fragmentation in those cells (Figs. 3A and 3B). Although some larger antral follicles showed cells with fragmented DNA (Fig. 3D), most follicles of that size did not show DNA fragmentation. In smaller follicles of antibody-treated rats, DNA fragmentation was apparent in granulosa cells throughout the follicle (Figs 3A and 3B) but cell death in larger follicles was scattered and tended to occur more predominantly in the cells lining the antral cavity. Occasionally, cell death was observed in follicles in the control ovaries but to much less extent than in the antibody-treated group (Fig. 3E). Cell death was rarely observed in theca cells of either treatment group. Interestingly, surface epithelial cells displayed fragmented DNA, and this was seen in both control and treated ovaries.

III. Induction of Granulosa Cell Apoptosis by Anti-eCG Antibody

Granulosa cells isolated from control and antibody-treated rat ovaries were stained for examination of nuclear morphology, or were frozen for determination of apoptotic DNA production. Nuclear blebbing, chromatin condensation, and increased fluorescence intensity, morphological indexes of apoptotic nuclei (Arends *et al.*, Am. J. Physiol. 136: 593-608, 1990; Wyllie *et al.*, Int. Rev. Cytol. 68: 251-356, 1980), were evident in the some nuclei of granulosa cells isolated from control ovaries (2%), but significantly more apoptotic nuclei were observed in granulosa cells from antibody-treated rats (Fig. 4B, arrowheads; Fig. 5B). DNA isolated from granulosa cells was end-labelled with [^{32}P]dCTP, and resolved by electrophoresis and autoradiography. The DNA from antibody-treated, but not control, rat granulosa cells displayed the characteristic ladder pattern of fragmentation indicative of apoptosis (Fig. 5C). A time course of low molecular weight (>15 Kbp) DNA production in granulosa cells from control and antibody-treated rats revealed that low molecular

weight DNA production was significantly increased by 1 hour after anti-eCG antibody injection, and was further increased by 6 and 24 hours (Fig. 6).

DISCUSSION OF MODEL

The present study demonstrates that anti-eCG antibody induces apoptosis *in vivo* in small to medium sized antral follicles of eCG-primed rat ovaries. Apoptosis was detectable by 1 hour after antibody injection as a significant increase in low molecular weight DNA which could be visualized as DNA ladders on electrophoretic gels. TUNEL and viable staining with acridine orange demonstrated that the apoptotic DNA degradation was occurring primarily in granulosa cells of small to medium sized antral follicles. Hence, gonadotropin prevents apoptosis in granulosa cells during the critical early stages of granulosa cell differentiation, suppressing atresia and allowing the follicle to develop to the preovulatory stage.

We observed classic signs of atresia in histological sections of ovaries of the antibody-treated rats. These included thinning of the granulosa layer, partial detachment from the basal lamina, and the occurrence of cells or debris in the follicle antrum. The reduced ovarian weight observed in these animals is also consistent with the induction of atresia by the antibody. Both *in situ* TUNEL labelling and acridine orange staining identified dead cells in small to medium sized antral follicles. Although TUNEL has been employed for this purpose in other studies, this is the first report of acridine orange staining to detect cell death in the ovary. The technique is simple and provides rapid results and avoids any possible artifacts that may arise as a result of fixation and processing of tissues. One caveat of TUNEL and acridine orange staining, however, is that it may not distinguish between necrotic and apoptotic cell death. We therefore confirmed the occurrence of apoptotic cell death by morphological analysis of Hoescht stained granulosa cells from antibody-treated rats. Typical morphological signs of apoptosis were observed in these granulosa cells from antibody-treated rats. In addition, necrotic DNA degradation results in a smear of

DNA during electrophoretic analysis, but this was not observed in our studies. Hoescht staining of granulosa cells identified apoptosis in approximately 12% of the population in antibody-treated rats (c.f. 2% in controls), a finding in good agreement with the identification of atresia by pyknotic nuclei described by Byskov (Byskov, A.G., J. Reprod. Fertil. 37: 277-285, 1974). It is possible that both Hoescht staining or pyknotic nuclei may underestimate the number of apoptotic cells because fragmentation may proceed very rapidly during apoptosis.

The rapid onset of apoptosis is one of the key features of this model.

Other signs of atresia following this drop in steroid production included elevated cAMP after 2 hour and subsequent decrease beyond 12 hours, pyknotic granulosa cells after 4-8 hours which become maximal by 48 hours. and a decrease of 69% in ^{125}I-labelled FSH binding to granulosa cells by 24 hours. The decline in estrogen production is a consistent feature of atresia in different species (Greenwald and Terranova, "Follicular selection and its control", The Physiology of Reproduction, eds. E. Knobil and J. Neill, Raven Press, NY, pp. 387-435, 1988). Our finding that apoptotic DNA degradation (endonuclease activation) is evident within 1 hour of antibody treatment is consistent with the reported rapid decline in estrogen levels, although it is not known, at this time, whether the drop in estrogen levels is a cause or effect of granulosa cell apoptosis. The preferential cleavage of transcriptionally active chromatin may be one function of endonuclease activity during apoptosis, and selective decreases in aromatase mRNA, which could result in cessation of androgen aromatization, have been demonstrated in atretic porcine follicles. Estrogen suppresses apoptosis in immature follicles *in vivo*, and androgen enhances apoptosis after estrogen withdrawal. The cessation of aromatase gene transcription after endonuclease activation could conceivably cause estrogen levels to decline and androgen levels to increase, but it is not known whether one or both of these effects on steroid production mediates the progression of apoptosis in granulosa cells.

Both FSH and (Luteinizing Hormone) LH activity are present in eCG and therefore the induction of apoptosis by eCG withdrawal could reflect the cessation of either FSH or LH stimulation, or both. In small follicles, FSH binds exclusively to granulosa cells and LH binding is restricted to the theca (Camp *et al.*, Mol. Endocrinol. 5: 1405-1417, 1991; Borolussi *et al.*, Cell Tiss. Res. 197: 213-226, 1979), and apoptosis occurs in the granulosa but not the theca after eCG withdrawal. FSH, but not LH, is a dominant survival factor in cultured early antral follicles, and apoptosis is observed primarily in follicles of this size after antibody treatment *in vivo*, suggesting that it is the withdrawal of FSH stimulation that induces apoptosis after antibody treatment. Although FSH withdrawal can induce apoptosis in preovulatory follicles *in vitro*, we did not observe extensive apoptosis in larger follicles after eCG withdrawal *in vivo*. It is possible to completely remove FSH stimulation *in vitro* by culturing follicles in serum free media, but this is not the case *in vivo*. It is most likely that during antibody treatment, the levels of eCG decrease to a level below that required for small antral follicle survival but sufficient amounts of the gonadotropin remain to stimulate larger follicles, which have more receptors and are more responsive to small amounts of gonadotropin.

It is not clear from these data whether the actions of FSH to suppress granulosa cell apoptosis are direct. FSH stimulates the production of estrogen, EGF receptor, IGF-1, and cAMP in ovarian cells, and these agents have been demonstrated to inhibit apoptosis in ovarian follicles. Activan, which interacts with FSH to modulate follicle development, has also been implicated as a survival factor in early antral follicles. In addition, FSH can regulate the production of cytokines, such as IL-6, which has been implicated in apoptosis of granulosa cells (Gorospe and Spangelo, Endocr. J. 1: 3-9, 1993; Gorospe and Spangelo, Biol. Reprod. 48: 538-543, 1993).

The results of this aspect of the study suggests that gonadotropin withdrawal by the anti-eCG antibody is an excellent model because it induces apoptosis, and therefore atresia in follicles commonly seen to undergo atresia under normal

physiological conditions. Gonadotropin withdrawal *in vivo* (with anti-eCG antibody) significantly decreased ovarian weight, increased incidents of follicular atresia and granulosa cell apoptosis, as evident by membrane blebbing and nuclear fragmentation (Figs. 1A-6). In addition, the induced withdrawal of gonadotropin allows more control over the timing of follicle atresia than can be accomplished with a single injection of eCG and subsequent eCG metabolism after 3 days of follicle growth. The ability to induce atresia in small to medium sized follicles in a controlled fashion, and rapid onset of apoptosis following gonadotropin withdrawal, make this model ideal for the study of signalling mechanism(s) and early events in granulosa cell apoptosis and follicle atresia *in vivo*.

2. ROLE OF IAPs AND NAIP IN THE CONTROL OF OVARIAN FOLLICULAR ATRESIA

We have studied the role and regulation of granulosa cell apoptosis and the participation of IAPs in the regulation of ovarian follicular atresia, particularly in the context of ovarian dysfunction, and the development of potential novel therapeutic interventions.

The inhibitor of apoptosis proteins (IAPs) constitute a family of highly conserved apoptosis suppressor proteins that was originally identified in baculoviruses. Although IAP homologs have been recently identified and demonstrated to suppress apoptosis in mammalian cells, their expression and role during follicular development and atresia are unknown. This part of the present study was conducted to address these questions. Using *in vivo* models for the induction of follicular development and atresia in immature rats, it was possible to compare the immunolocalization of two members of the IAP family, X-linked Inhibitor of Apoptosis Protein (XIAP) and Human Inhibitor of Apoptosis Protein-2 (HIAP-2), at defined stages of follicular maturation and to relate the differences observed with those of follicular cell proliferation and apoptosis, as determined by proliferating cell

nuclear antigen (PCNA) immunohistochemistry and *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labelling (TUNEL), respectively. In addition, granulosa cell DNA and proteins were assessed for apoptotic fragmentation by 3'-end labelling/agarose gel electrophoresis (DNA ladder formation), and HIAP-2 and XIAP protein content by Western blot analysis. HIAP-2 and XIAP expression in both granulosa and theca cells increased with follicular maturation, reaching maximal levels at the antral stage of development. The immunoreactivity for PCNA, XIAP and HIAP-2 decreased markedly in atretic (TUNEL-positive) follicles at the small to medium sized antral stage of development, suggesting follicular atresia is associated with decreased granulosa cell IAP protein content and decreased proliferation. Atresia was also associated with a change in the intracellular distribution of IAPs in granulosa cells. Biochemical analysis of DNA fragmentation (DNA ladder) in granulosa cells from preantral and early antral follicles indicates extensive apoptosis which was associated with minimal IAP protein content. Gonadotropin treatment increased HIAP-2 and XIAP protein content and suppressed apoptosis in granulosa cells, resulting in the development of follicles to the antral and preovulatory stages. In addition, gonadotropin withdrawal induced apoptotic DNA fragmentation in granulosa cells in early antral and antral follicles, which is accompanied by a marked decrease in HIAP-2 and XIAP expression. These data suggest that IAPs are involved in the suppression of granulosa cell apoptosis by gonadotropin in small to medium sized antral follicles and play an important role in determining the fate of the cells, and thus also the eventual follicular destiny (atresia versus ovulation).

MATERIALS AND METHODS

I. Materials

10% neutral buffered formalin, xylene, ethanol, paraffin, acetic acid, EDTA and $MgCl_2$ were purchased from BDH (Toronto, ON). Ethidium bromide, Diethylstilbestrol (DES), equine chorionic gonadotropin (eCG), human chorionic

gonadotropin (hCG), agarose, Hoescht 33248, acridine orange, Tris, PBS, protease K, Phenylmethylsulifonyl fluoride (PMSF), goat serum and eCG were from Sigma (St Louis, MO). Klenow enzyme, ECL Western blotting detection kit and [α^{32}P]-ddATP (3000 Ci/mmo) were obtained from Amersham (Arlington Heights, IL). Medium M199 and normal rabbit serum were from Gibco/BRL (Burlington, ON). Zeta-probe blotting membrane, trans-blot supported nitrocellulose membrane, acrylamide (electrophoresis grade), N,N'-methylene-bis-acrylamide, ammonium persulfate, tetramethylethylene diamine, dithiothreitol, glycine, SDS-PAGE prestained molecular weight standards (low range), and Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA). X-ray films were from Eastman Kodak Company (Rochester, NY). Positively charged slides were from Fisher (Probe On Plus, PA). The anti-eCG antibody was prepared by rabbit immunization with eCG by standard molecular biology techniques. Rabbit polyclonal anti- XIAP and HIAP-2 antibodies were prepared by immunization with human XIAP and HIAP-2 GST fusion protein. The PCNA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

II. Animal Treatment Groups

The induction of ovarian follicular development and atresia was generally done as described above. Specifically, for the induction of follicular development, immature female Sprague-Dawley rats at 23-24 days of age (weighing 50-60 g, Charles River Canada, Montreal, Quebec, Canada) received injection of either diethylstilbestrol (DES, 1 mg/day, s.c. for three consecutive days; animals sacrificed 24 hours after the last injection) or equine chorionic gonadotropin (eCG, 15 IU, i.p.; animals sacrificed 48 hours post-eCG). A third group of animals was injected with eCG as described earlier, but was also injected with human chorionic gonadotropin (hCG, 15 UI, i.p. 48 hours after eCG injection; animals sacrificed 8 hours post-hCG). These hormonal treatments synchronize ovarian follicular development at

predominantly the preantral/early antral follicles, small to medium-sized antral and preovulatory stages. and provides high yields of granulosa cells which are largely apoptotic, differentiated and luteinized, respectively .

For the induction of follicular atresia, 23-24 day-old immature female rats were injected with eCG (15 IU, s.c.) and 24 hours later with either 100 µL normal (pre-immune) rabbit serum or 100 µL anti-eCG antibody (1:10 in saline; i.p.). At the indicated times after serum or antibody injection (1 hour or 24 hours post-injection), animals were sacrificed and their ovaries were excised, cleared of adhering fat, weighed, and either fixed in 10% neutral buffered formalin for histology or placed in medium M199 for viable straining and granulosa cell collection. Granulosa cells were harvested by follicular puncture and centrifugation. While DES treatment provided predominantly preantral and early antral atretic follicles (as observed in naturally occurring immature ovaries), this gonadotropin withdrawal treatment induced atresia at the early antral and small to medium sized antral stages of development (as found in ovaries of naturally occurring cycling rats).

The animals were fed prolab RMH 4018 (AGWAY Inc., C.G., Syracuse, New York) and water ad libitum. A 14 hour/10 hour light/dark cycle was maintained with light cycle initiated at 06:00 hours. Ovaries were excised for immunohistochemistry for IAPs and proliferation cell nuclear antigen (PCNA), and in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labelling (TUNEL) of apoptotic cells. In addition, granulosa cells from each group of animals were harvested by follicle puncture as previously described (Karakji and Tsang 1995, Biol Reprod. 52: 411- 418, 1995), washed (900 x g, 10 minutes) and resuspended in 10 mM HEPES buffer (pH 7.4) containing 1 mM EGTA and 2 mM PMSF

III. Histology and TUNEL Labelling

Histology was performed as described above.

For *in situ* 3'-end labelling (TUNEL), the paraffin embedded sections (4 μm) were first deparaffinized and dehydrated. The TUNEL labelling was then done generally as described above. Briefly, paraffin embedded whole ovarian sections (4-5 μm) were mounted on positively charged slides, deparaffinized, hydrated, and treated with proteinase-K (10 μg/ml in 20 mM Tris and 2 mM $CaCl_2$, pH 7.4; 37°C, 30 minutes) and then washed thoroughly for 15 minutes in Tris buffer (100 mM Tris and 150 mM NaCl, pH 7.5), followed by immersion in methanol containing 0.3 % H_2O_2 (room temperature, 20 minutes) to inhibit endogenous peroxidase activity. After rinsing in distilled water for 15 minutes, the sections were soaked in the terminal transferase (TdT) buffer (25 mM Tris-HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride, 250 μg/ml bovine serum albumin, pH 6.6; 15 minutes), and then incubated in 50 μl of TdT buffer containing 10 U TdT and 1 nmol biotinylated 16-dUTP in a humidified chamber (37°C, 60 minutes). The reaction was stopped by soaking sections in 2X SSC (300 mM NaCl, 30 mM sodium citrate), followed by washing in phosphate buffered saline (PBS; room temperature, 15 minutes). The biotinylated dUTP molecules incorporated into nuclear DNA were visualized by incubation with horseradish peroxidase-conjugated streptavidin (1:100; room temperature, 30 minutes). After further washing in PBS (15 minutes), the sections were immersed for 10 minutes in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.3 mg/ml diaminobenzidine tetrahydrochloride (DAB), 0.65 mg/ml sodium azide, 10 mM imidazole, and 0.003 % H_2O_2 (peroxidase coloring reaction). The nuclei were counterstained with 5 % methyl green buffered with 0.1 M veronal acetate, pH 4.0. In the negative control slides, TdT enzyme or biotinylated 16-dUTP were omitted in labelling reactions.

IV. Identification of Cell Death

Viable staining of cells, nuclear staining, and DNA ladder quantification was performed as described above.

DNA fragmentation analysis was performed as follows. DNA was extracted and labelled as previously described (Tilly and Hsuch, J. Cell. Physiol. 154: 519-526, 1993). To 3'-end label DNA, 1 µg of DNA was incubated with 25 U terminal deoxynucleotidyl transferase (TdT) and 5 µCi [α^{32}P]-ddATP (3000 Ci/mmol) in 50 µl of 200 mM sodium cacodylate, 5 mM cobalt chloride, 250 µg/ml bovine serum albumin, 25 mM Tris-HCl, pH 6.6 (37°C, 60 minutes) and the reaction was terminated by the addition of 5 ml of 250 mM EDTA, pH 8.0. Unincorporated radionucleotide in the reactions were removed by the addition of 0.2X volume 10 M ammonium acetate and 3X volume ice-cold 100 % ethanol, followed by incubation with 50 mg yeast tRNA (-70°C, 60 minutes). The nucleic acid was collected by centrifugation (14,000 x g; 4°C, 20 minutes), resuspended in buffer, and re-precipitated with ethanol. The DNA was again pelleted by centrifugation, washed with 0.25 ml ice-cold 80 % ethanol, and allowed to air-dry. Samples were resuspended in TE buffer. The labelled samples were resolved by 1.8 % agarose gel electrophoresis. The gel was dried (3 hours) and exposed to a Bio-Rad phosphorimager screen to densitometrically quantify low molecular weight DNA (< 4 Kbp) and subsequently exposed to X-ray film at -80°C.

V. Western Blot Analyses for IAPs

Protein extracts were prepared from granulosa cells sonicated (8 sec/cycle, 3 cycles) on ice in sucrose buffer (0.25 M sucrose, 0.025 M NaCl, 1 mM EGTA and 15 mM Tris-CH1 pH 6.8, supplemented with 1 mM PMSF, 2 µg/ml of leupeptin, and 5 µg/ml of aprotinin) or 10 mM HEPES buffer (pH 7.4) containing 1 mM EGTA and 2 mM PMSF.. The sonicates were centrifuged at 13,000 x g for 10 minutes, and the supernatants were then collected and stored at -20°C until electrophoretic analyses were performed. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories). Equal amounts of proteins (10-80 µg, depending on individual experiments) were resolved by one-dimensional SDS-PAGE, and

electrophoretically transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat milk, and subsequently incubated with rabbit polyclonal antibody directed toward XIAP, HIAP-2, or PCNA antibody diluted in TBST (10 mM Tris-buffered saline, 0.1% Tween-20, pH 7.5) containing 5% non-fat milk. The rabbit polyclonal anti-IAP antibodies were as follows: anti-human HIAP-2ΔE (960529, used at 1:1000 dilution), or anti-human XIAP, used at 1:1000 dilution. An ECL kit was used to visualize immunopositive protein (Amersham).

VI. Immunohistochemical Analyses of IAPs.

Paraffin embedded whole ovarian sections (4 μm) were incubated for 15 minutes in 3 % H_2O_2, and rinsed thoroughly with PBS (3 washes for 15 minutes each). The sections were blocked with 1.5 % normal goat serum in PBS (room temperature, 1 hour) to suppress non-specific binding of IgG, and then incubated (room temperature, 45 minutes) with rabbit polyclonal anti-human XIAP (1 μg/ml), HIAP-2 (2 μg/ml), or PCNA antibodies in 1 % blocking serum in PBS. After washing with PBS (3 washes for 15 minutes each), the sections were incubated with biotin-conjugated goat anti-rabbit IgG (1:200 in PBS; room temperature, 1 hour), followed by avidin-biotin-peroxidase complex (room temperature, 1 hour) from a Vector ABC Elite Kit (Vector Laboratories Inc., Burlingame, CA). They were again washed with PBS (3 washes for 15 minutes each) and incubated with diaminobenzidine (0.1 μg/ml, DAB) solution (2-5 minutes). The nuclei were counterstained with hematoxylin or methyl green. As a negative control, rabbit IgG (1 μg/ml) was applied to primary antibody reaction in this experiment.

VII. Statistics Analysis

Results were expressed as the mean ± SEM of three to five experiments. Statistical analysis were carried out by one- or two-way Analysis of Variance.

Significant differences between treatment groups were determined by the Tukey test. Statistical significance was inferred at $P<0.05$.

3. RESULTS

To look at the correlation of IAP protein expression with ovarian follicle development, eCG was injected into rats to induce ovarian follicular development and suppressed granulosa cell apoptosis.

Studies on the co-localization of apoptotic cells, XIAP, and HIAP-2 in ovarian sections from rats undergoing gonadotropin withdrawal *in vivo* demonstrated atretic follicles, which stain positive by TUNEL labelling, are negative for both XIAP and HIAP-2 expression (Fig. 7). Hence, XIAP and HIAP-2 were highly expressed in normal (healthy follicles) but not apoptotic (atretic follicles) granulosa cells.

In a second, more comprehensive experiment, XIAP and HIAP-2 were found immunolocalized in both granulosa and theca cells in the rat ovary during follicular development, using polyclonal antibodies against human XIAP and HIAP-2, respectively (Figs. 8A-8P). HIAP-2 and XIAP were found in the cytoplasm and nuclei of granulosa cells from healthy follicles (Figs. 9C and 9E, respectivley). *In situ* TUNEL and immunohistochemistry for PCNA (an auxiliary protein of DNA polymerase α that is highly expressed at G1/S interphase), performed to study if and how XIAP and HIAP-2 expression relates to granulosa cell apoptosis and proliferation during follicular development and atresia, indicates that HIAP-2 and XIAP were expressed in both granulosa and theca cells in healthy follicles (TUNEL negative; Figs. 8A, 8B, and 8C), when the cells were proliferatively most active (PCNA positive: Figs. 8E, 8F, and 8G). Table 1 shows a summary of TUNEL and immunohistochemistry of PCNA, HIAP-2, and XIAP at different stages of follicular development in rat ovaries.

Table 1

	Preantral Follicles				Early Antral Follicles				Antral Follicles			
	Healthy		Atretic		Healthy		Atretic		Healthy		Atretic	
	GC	TC	GC	TC	GC	TC	GC	TC	GC	TC	GC	TC
TUNEL	-	-	+	-	-	-	+++	-	-	-	++	-
PCNA	+	++	+	-	+	++	+	-	++	+++	+	-
HIAP-2	+	++	+	+	+	++	+	+	++	+++	+	-
XIAP	+	++	+	+	+	++	+	+	++	+++	+	-

Signal intensity: -, weak; +, moderate; ++ intense; +++, very intense
GC: granulosa cells, TC: theca cells

As summarized in Table 1, HIAP-2 and XIAP were less abundant in granulosa cells from preantral (*e.g.*, "H" in Fig. 8A) and early antral (*e.g.*, "H" in Fig. 8B) follicles where follicular atresia is frequently observed (HIAP-2, Figs. 8J, 8J, 8K, and 8L; XIAP, Figs. 8M, 8N, 8O, and 8P). Consistent with our present findings (Figs. 8A, 8B, 8C, and 8D) that granulosa but not theca cells undergo apoptosis, HIAP-2 and XIAP expression were greater in theca than in granulosa cells from preantral, early antral, and antral follicles (HIAP-2, Figs. 8I, 8J, 8K, and 8L; XIAP, Figs. 8M, 8N, 8O, and 8P). Oocytes (Figs. 8K and 8O) and ovarian surface epithelial cells (Figs. 8L and 8P) also stained positively for HIAP-2 and XIAP.

Atretic follicles ("A" in Figs. 8A-8P) were evident by the presence of TUNEL-positive granulosa cells, uneven granulosa cell layers and loosely attached granulosa cells which could occasionally be detected within the antral cavity (Figs. 8A-8P). Compared to those from healthy follicles, granulosa cell PCNA staining intensity in atretic follicles at the preantral and early antral stages was low (compare "H" to "A" in Figs. 8E and 8F). However, granulosa cell proliferation was most active in healthy, small-to-medium sized antral follicles, as indicated by intense PCNA staining (Fig. 8G). PCNA protein expression decreased dramatically in atretic follicle at this stage of development, confirming the well established notion that follicular atresia is

accompanied by decreased granulosa cell proliferation (Figs. 8G and 8H). Although parts (right side) of the atretic antral follicle (Fig. 8G) appeared somewhat stained for PCNA, close examination at higher magnification demonstrated that, in contrast to that in healthy follicles where PCNA was nucleus-specific, this might be artifactual as the heavy immunostaining of this atretic follicle was localized outside the nuclei (Figs. 9A and 9B). Similar developmental patterns were observed in granulosa cell IAP expression, where significant decrease in XIAP (Figs. 8M, 8N, 8O, and 8P) and HIAP-2 (Figs. 8I, 8J, 8K, and 8L) abundance was only evident in atretic follicles at the mid to late antral stages, suggesting a decrease in IAP expression may be associated with atresia in this follicle stage. In addition, there was a change in the intracellular distribution of IAPs in granulosa cells with the induction of atresia. Whereas granulosa cells from healthy antral follicles exhibited more intense HIAP-2 immunoreactivity in the nuclei than the cytoplasm, this IAP was predominantly localized in the extra-nuclear portion of the dying cells (detached from follicular wall) in atretic follicles (Figs. 9C and 9D). Although the intracellular localization of XIAP in the healthy follicles was similar to that of HIAP-2, a relatively higher proportion of XIAP immunoreactivity in granulosa cells of atretic follicles appeared to be present in the cytoplasm than in the nuclei (Figs. 9E and 9F). Interestingly, compared to healthy follicles, a decrease in theca cell IAP expression observed in atretic follicles (compare "H" to "A" in Figs. 8K. 8L, 8O, and 8P) was accompanied by decreased PCNA immunoreactivity irrespective of follicular maturation (Figs. 8E, 8F, 8G, and 8H; Table 1).

As shown in Fig. 10, HIAP-2 protein expression increases with follicle maturation. Granulosa cells from early (E) stage follicles (preantral and early antral follicles) showed little or no HIAP-2 expression by Western blot analysis. In contrast, granulosa cells from mid (M) stage follicles (small to medium-sized antral follicles) showed moderate HIAP-2 expression, which increases in granulosa cells from late (L)

stage follicles (preovulatory follicles). Hence, follicular maturity was associated with increased HIAP-2 protein expression (Western blot; Fig. 10).

Further experiments were performed to confirmed the results from immunohistochemistry and determine quantitatively whether granulosa cell IAP expression may be inversely related to apoptosis. Granulosa cells were isolated from follicles in DES (preantral and early antral stages)-, eCG (mid to late antral stages)-, and hCG (preovulatory stage)-treated rats. IAP expression and DNA fragmentation were analyzed by Western blot analysis and 3'-end DNA labelling/agarose gel electrophoresis, respectively. As shown in Figs. 11A and 11B, granulosa cell HIAP-2 (68 kDa) expression was minimal in the preantral and early antral stages of follicular development and increased with follicular maturation ($p < 0.05$). A similar pattern of developmental expression was also observed for XIAP (55 kDa), although the difference between the stages failed to meet statistical significance ($p > 0.05$; Fig. 11D). IAP expression was inversely correlated with granulsoa cell DNA fragmentation (Figs. 11F and 11F). Apoptosis was most evident in the DES group when HIAP-2 abundance was lowest (Figs. 11B, 11F).

Gonadotropin is an important survival factor for the developing follicles to escape atresia and reach the preovulatory follicle stage (Byskov, A.G., *supra*; Billig *et al.*, *supra*; Chun *et al.*, *supra*; Hirshfield and Midgley, *supra*; Schwartz N.B., *supra*). Gonadotropin withdrawal induces granulosa cell apoptosis and follicular atresia (Chun *et al.*, *supra*). In the present study, we have employed a well characterized gonadotropin withdrawal immature rat model to further study the role of IAPs in follicular atresia. Rats were injected with eCG and 24 hours later with either anti-eCG antibody or normal rabbit serum (control) and ovaries were removed 1 and 24 hours (*i.e.*, 25 and 48 hours after eCG injection) thereafter for granulosa cell isolation. As shown in Figs. 12A-12D, HIAP-2 and XIAP expression was induced by eCG *in vivo* in a time-dependent manner (Figs. 12A, 12B, 12C, and 12D). Withdrawal of gonadotropin support in the eCG-primed animals with an anti-eCG antibody markedly

suppressed HIAP-2 (Figs. 12A and 12B) and XIAP (Figs. 12C and 12D) expression at 24 hours, compared to respective controls. These responses were accompanied by a marked increase in granulosa cell apoptosis as evident by the appearance of extensive (8-fold increase) DNA fragmentation in the antibody group compared to its respective control (Figs. 12E and 12F).

Suppression of granlosa cell HIAP-2 and XIAP protein expression is very rapid. As shown in Figs. 13A and 13B, Western blotting analysis indicates that gonadotropin withdrawal (by addition of anti-eCG) also markedly suppressed granulosa cell HIAP-2 (Fig. 13A) and XIAP (Fig. 13B) protein content within 1 hour.

4. SIGNIFICANT OBSERVATIONS AND CLINICAL IMPLICATIONS

The present studies demonstrate that gonadotropin stimulation induces follicular development, which is accompanied by marked decrease in the incidence of granulosa cells apoptosis and stimulated expression of various IAP genes. Conversely, withdrawal of gonadotropin support resulted in suppression of IAP gene expression, increase in granulosa cell apoptosis and the incidence of follicular atresia. These findings clearly demonstrate that the IAP genes are closely associated with the regulation of granulosa cell apoptosis and ovarian follicular atresia and are prime sites of gene targeting in the development of novel drugs for the treatment of infertility associated with enhanced follicular atresia (*e.g.*, premature ovarian failure, polycystic ovarian diseases), as well as in the recruitment of follicles into the developing/ovulatory pool in patients undergoing *in vitro* fertilization and embryo transfer as well as those resistant to gonadotropin induction.

We have demonstrated here that eCG induced XIAP and HIAP-2 expression and suppressed granulosa cell apoptosis *in vivo*. Withdrawal of gonadotropin support attenuated the eCG-induced granulosa cell IAP expression and markedly increased apoptotic DNA fragmentation in early antral and antral follicles. Our results raise the interesting possibility that induction of IAP expression may be an important

mechanism underlying the anti-atretogenic action of gonadotropin in the ovary. In addition, the current demonstration that whereas HIAP-2 and XIAP are mainly localized in the nuclei of granulosa cells from healthy follicles, the induction of granulosa cell apoptosis during follicular atresia appeared to be associated with a change in the intracellular distribution of these protein, with predominant localization being in the cytoplasm. The physiologic significant of these changes has yet to be elucidated.

In the ovary, as in other cell systems, cell death inducers (*e.g.*, Fas) and survival factors, *e.g.*, Bcl-xL and IAP (as demonstrated herein), are constitutively present (Hakuno *et al.*,. Endocrinology 137:1938-1948, 1996; Auyeung *et al.*, XIth Ovarian Workshop: Ovarian Cell Growth, Apoptosis and Cancer, London, Ontario, July 24-26, 1996; Johnson *et al.*, Endocrinology 137: 2059-2066, 1996; Martin and Green, Cell 82: 349-356, 1995) and the fate of the granulosa cell (survival vs. apoptosis) is determined by the balance of these opposing activities. The survival of granulosa cells in follicles that escape atresia and selected to ovulate in each reproductive cycle may occur through up-regulation of the survival factors and/or removal of the cell death inducers by an appropriate stimuli. It is possible that by inducing HIAP-2 and XIAP overexpression in granulosa cells, FSH is able to tilt the balance towards cell survival, and thus follicular growth and ovulation. Alternatively, our studies have demonstrated that whereas gonadotropin suppressed granulosa cell Fas and Fas ligand expression in antral and preovulatory follicles in immature rats, gonadotropin withdrawal by treatment of the eCG-primed animals with anti-eCG anti-serum (as in the present studies) resulted in the overexpression of these cell death factors and induced follicular atresia (Auyeung *et al.*, *supra*). The relative importance of these cellular changes and the interactions of their pathways in the induction of granulosa cell apoptosis remains to be determined.

Apoptosis in atretic follicles in most mammals studied to date are confined to granulosa cells (Tilly *et al.*, Endocrinology 129: 2799-2801, 1991; Baker T. G., Proc.

R. Soc.. London Ser. B. 158: 417-433, 1963). We observed that. irrespective of the stage of follicular maturation, theca cells in all atretic follicles were TUNEL-negative. Interestingly, XIAP and HIAP-2 were highly expressed in theca cells and their immunoreactivities in preantral and early antral follicles were much higher than those in granulosa cells. This phenomenon may be one of the mechanisms in place to prevent theca cells from undergoing apoptosis. Alternatively, IAPs may be involved in other as yet undetermined physiological process such as theca cell differentiation.

The mechanism(s) by which IAPs interact with established cell death pathways to regulate apoptosis is poorly understood. Recent studies reported that HIAP-2 is able to interact with signalling component of both tumour necrosis factor (TNF) receptor I and II (TNFR1 and TNFR2) pathways (Rothe *et al.*, Cell 83: 1243-1252, 1995; Shu *et al.*, Proc. Natl. Acad. Sci. USA 93: 13973-13978, 1996). While TNFR1 signals mainly for cytotoxicity (Smith *et al.*, Cell 76: 959-962, 1994), TNFR2 has been implicated in cell proliferation or survival (Tartaglia *et al.*, J. Immunol. 151: 4637-4641, 1993). Using biochemical purification and subsequent molecular cloning method, HIAP-2 has been shown to be a component of the of the TNFR-2 signaling complex, in which the BIR motif-containing domain interacts with TRAF2 (Rothe *et al.*, Cell 83: 1243-1252, 1995). TRAF1 and TRAF2 have been shown to interact with a region within the cytoplasmic domain of TNFR2 required for signal transduction (Rothe *et al.*, Science 269: 1424-1427, 1995). Another report has indicated that HIAP-2 is one of the component of the "survival complex" consisting of HIAP-2, TRAF2 and the TNFR1 associated death domain protein (TRADD), and that this complex is formed prior to TNFR1 stimulation (Smith *et al.*, *supra*). Precisely how HIAP-2 modulate the multiple and overlapping signal transduction pathways of the two TNF receptor subtypes is unknown. The possibility that HIAP-2 may be inhibitory in one (TNFR1) and stimulatory in another (TNFR2) is intriguing and can not be excluded. In the latter context, a recent study by Lee *et al.* (J. Exp. Med. 185: 1275-1285, 1997) has suggested that human IAPs, via interaction with TRAF2,

facilitate TNFα-induced cell proliferation. In our study, HIAP-2 expression was higher in follicles where granulosa and theca cells were proliferatively active (PCNA positive), thus also raising the possibility that HIAP-2 could play a role in the regulation of follicular cell proliferation in the ovary.

Moreover, recent study by Deveraux *et al.* (*supra*) has demonstrated that XIAP inhibits caspase-3, a "cell death" protease downstream on the TNFR1 pathway, and a caspase-7, thus suppressing apoptosis. Interestingly, we have found that granulosa cells from ovarian atretic follicles induced by eCG withdrawal exhibited considerably higher caspase-3 immunoactivity compared to those from healthy ones. It is thus possible that, in addition to its up-regulation during the induction of granulosa cell apoptosis by gonadotropin withdrawal, caspase-3 may be an additional point of regulation by IAP.

In summary, XIAP and HIAP-2 are expressed in rat granulosa and theca cells during follicular development, and the abundance of IAPs in granulosa cells is regulated during follicular development and atresia in an anti-apoptotic fashion. IAPs appear to be an intracellular protein family important in the "life" and "death" decision of granulosa cells during follicular selection and may play a critical role as a cell survival factor in the control of stage-specific follicular atresia during development.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such

departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

Claims

1. A method of decreasing apoptosis in an ovarian cell, said method comprising administering to said ovarian cell an apoptosis-inhibiting amount of an IAP polypeptide or fragment thereof.

2. A method of decreasing apoptosis in an ovarian cell, said method comprising administering a compound which increases biological activity of an IAP polypeptide.

3. A method of decreasing apoptosis in an ovarian cell, said method comprising administering to said ovarian cell an apoptosis-inhibiting amount of a caspase-inhibiting caspase polypeptide fragment.

4. A method of decreasing apoptosis in an ovarian cell, said method comprising administering to said ovarian cell a compound which decreases biological activity of a caspase polypeptide.

5. The method of claim 1, 2, 3, or 4, wherein said ovarian cell is in a mammal.

6. The method of claim 5, wherein said mammal is a human.

7. The method of claim 5, wherein said mammal is diagnosed as having female infertility or is predisposed to developing female infertility.

8. The method of claim 1, 2, 3, or 4, wherein said ovarian cell is a granulosa cell.

9. The method of claim 2, wherein said compound is nucleic acid encoding said IAP polypeptide.

10. The method of claim 4, wherein said compound is a caspase polypeptide antisense nucleic acid.

11. The method of claim 4, wherein said compound is an antibody which specifically binds a caspase polypeptide.

12. The method of claim 11, wherein said antibody is a caspase neutralizing antibody.

13. The method of claim 2 or 4, wherein said compound is gonadotropin.

14. The method of claim 1 or 2, wherein said IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

15. The method of claim 3, 4, 10, 11, or 12, wherein said caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

16. A method of inducing apoptosis in an ovarian cell, said method comprising administering to said ovarian cell an apoptosis inducing amount of an IAP-inhibiting IAP polypeptide fragment.

17. A method of inducing apoptosis in an ovarian cell, said method comprising administering a compound which decreases biological activity of an IAP polypeptide.

18. A method of inducing apoptosis in an ovarian cell, said method comprising administering to said ovarian cell an apoptosis inducing amount of a caspase polypeptide or fragment thereof.

19. A method of inducing apoptosis in an ovarian cell, said method comprising administering a compound which increases biological activity of a caspase polypeptide.

20. The method of claim 16, 17, 18, or 19, wherein said ovarian cell is in a mammal.

21. The method of claim 20, wherein said mammal is a human.

22. The method of claim 20, wherein said mammal is diagnosed as having ovarian granulosa cell hyperplasia.

23. The method of claim 16, 17, 18, or 19, wherein said ovarian cell is a granulosa cell.

24. The method of claim 17, wherein said compound is an IAP polypeptide antisense nucleic acid.

25. The method of claim 17, wherein said compound is an antibody which specifically binds to an IAP member protein.

26. The method of claim 25, wherein said antibody is an IAP neutralizing antibody.

27. The method of claim 16, 17, 24, 25, or 26, wherein said IAP is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

28. The method of claim 18 or 19, wherein said caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

29. A method of identifying a compound that modulates apoptosis in an ovarian cell, said method comprising:

(a) providing a cell expressing an IAP polypeptide; and

(b) contacting said cell with a candidate compound and monitoring the expression of said IAP polypeptide, an alteration in the level of expression of said IAP polypeptide indicating the presence of a compound that modulates apoptosis in an ovarian cell.

30. A method of identifying a compound that modulates apoptosis in an ovarian cell, said method comprising:

(a) providing a cell expressing a functional IAP polypeptide; and

(b) contacting said cell with a candidate compound and monitoring the biological activity of said IAP polypeptide, an alteration in the level of biological activity of said IAP polypeptide indicating the presence of a compound that modulates apoptosis in an ovarian cell.

31. A method of identifying a compound that modulates apoptosis in an ovarian cell, said method comprising:

(a) providing a cell comprising a reporter gene operably linked to a promoter from a gene encoding an IAP polypeptide;

(b) contacting said cell with a candidate compound; and

(c) measuring expression of said reporter gene, an alteration in said expression in response to said candidate compound indicating the presence of a compound that modulates apoptosis in an ovarian cell.

32. The method of claim 29, 30, or 31, wherein said cell is an ovarian cell.

33. The method of claim 29, 30, or 31, wherein said alteration is an increase indicating said compound decreases apoptosis.

34. The method of claim 29, 30, or 31, wherein said alteration is a decrease indicating said compound increases apoptosis.

35. The method of claim 29, 30, or 31, wherein said IAP polypeptide is selected from a group consisting of NAIP, HIAP-1, HIAP-2, and XIAP.

36. The method of claim 29, 30, or 31, wherein said ovarian cell is a granulosa cell.

37. A method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell, said method comprising:

(a) providing a cell expressing a caspase polypeptide; and

(b) contacting said cell with a candidate compound and monitoring the expression of said caspase polypeptide, an alteration in the level of expression of said caspase polypeptide indicating the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell.

38. A method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell, said method comprising:

(a) providing a cell expressing a functional caspase polypeptide; and

(b) contacting said cell with a candidate compound and monitoring the biological activity of said IAP polypeptide, an alteration in the level of biological activity of said polypeptide indicating the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell.

39. A method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell, said method comprising:

(a) providing a cell comprising a reporter gene operably linked to a promoter from a gene encoding a caspase polypeptide;

(b) contacting said cell with a candidate compound; and

(c) measuring expression of said reporter gene, an alteration in said expression in response to said candidate compound indicating the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell.

40. A method of identifying a compound that modulates IAP-associated apoptosis in an ovarian cell, said method comprising:

(a) providing a caspase polypeptide, said polypeptide isolated from a cellular extract;

(b) contacting said caspase polypeptide with a candidate compound; and

(c) measuring ability of said caspase polypeptide to cleave a caspase substrate, an alteration in said ability in response to said candidate compound indicating the presence of a compound that modulates IAP-associated apoptosis in an ovarian cell.

41. The method of claim 37, 38, 39, or 40, wherein said cell is an ovarian cell.

42. The method of claim 37, 38, 39, or 40, wherein said alteration is an increase indicating said compound increases apoptosis.

43. The method of claim 37, 38, 39, or 40, wherein said alteration is a decrease indicating said compound decreases apoptosis.

44. The method of claim 37, 38, 39, or 40, wherein said caspase polypeptide is selected from a group consisting of caspase-3 and caspase-7.

45. The method of claim 37, 38, 39, or 40, wherein said ovarian cell is a granulosa cell.

Fig. 1A

Fig. 1B

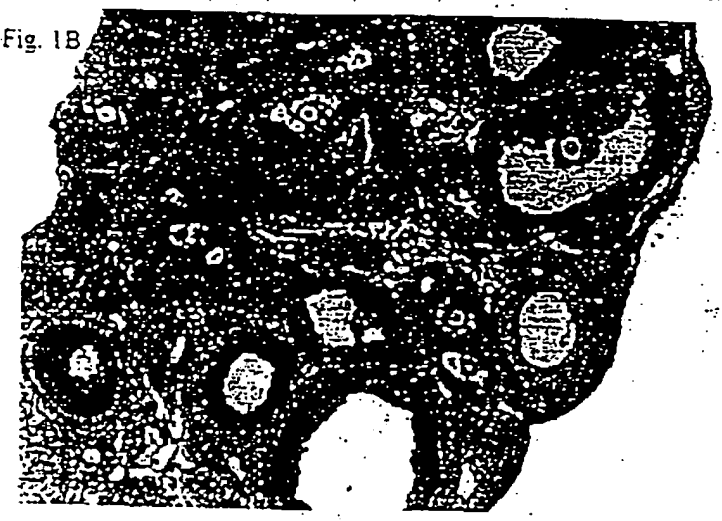


Fig. 2A

Fig. 2B

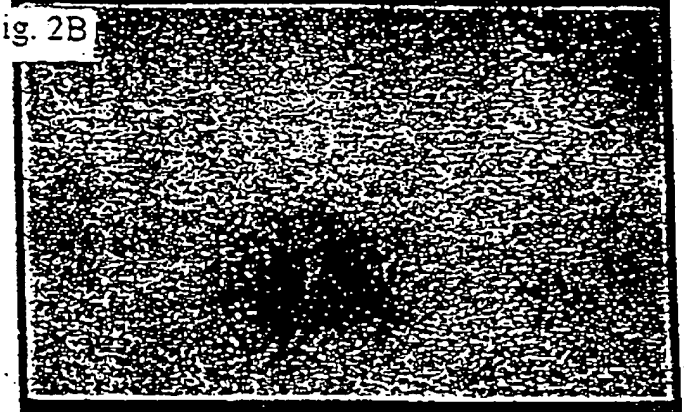
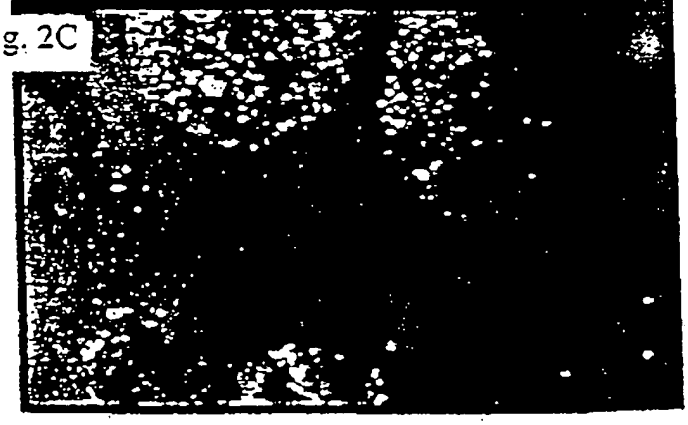


Fig. 2C



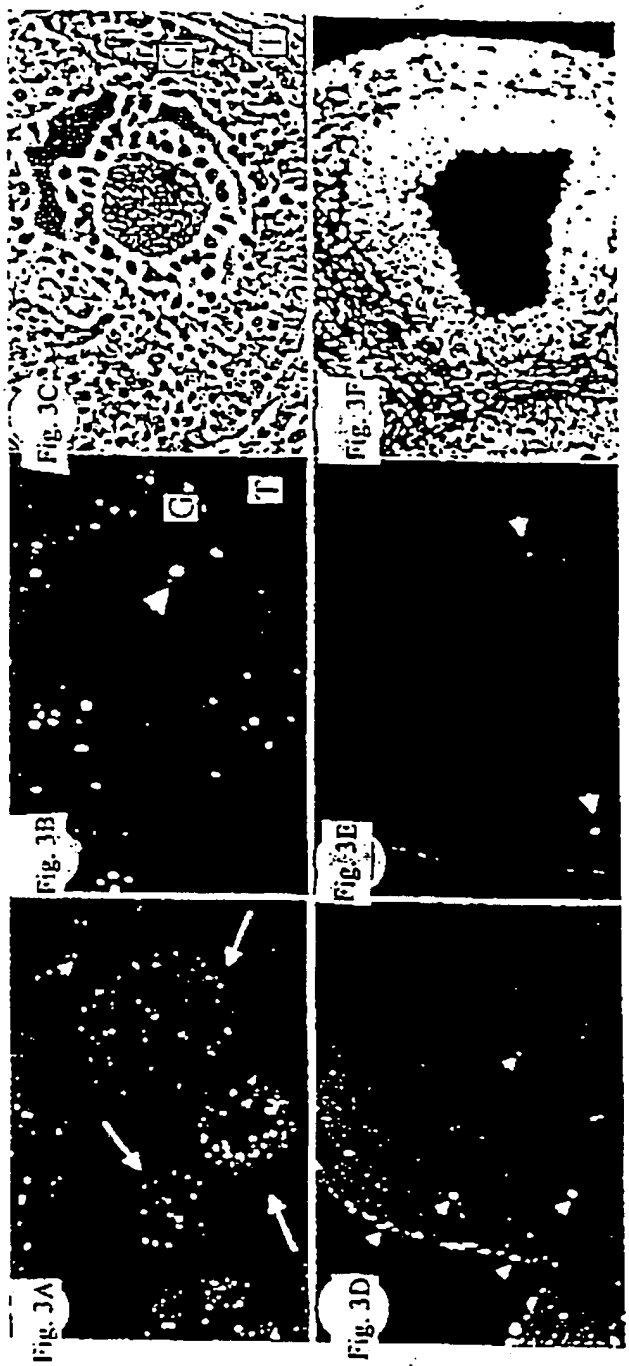

C
T
Fig. 3C
Fig. 3F
G
T
Fig. 3B
Fig. 3E
Fig. 3A
Fig. 3D

Fig. 4A

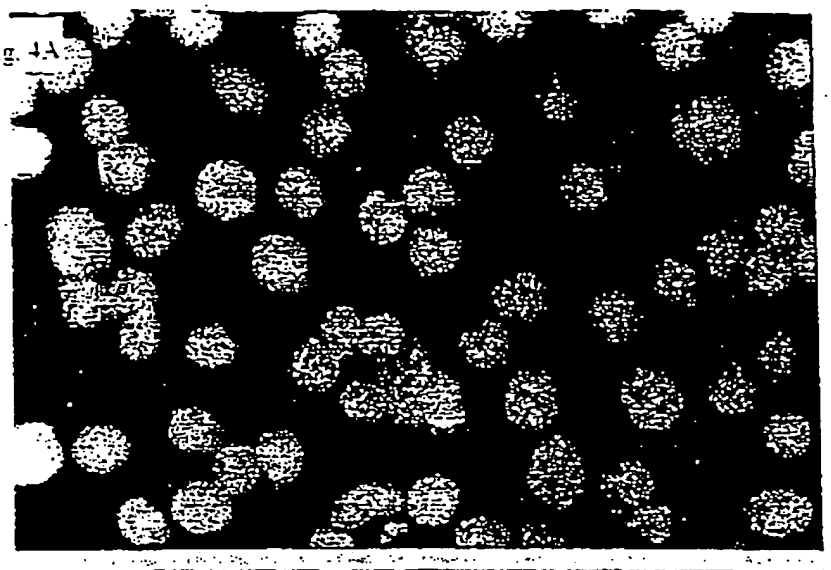
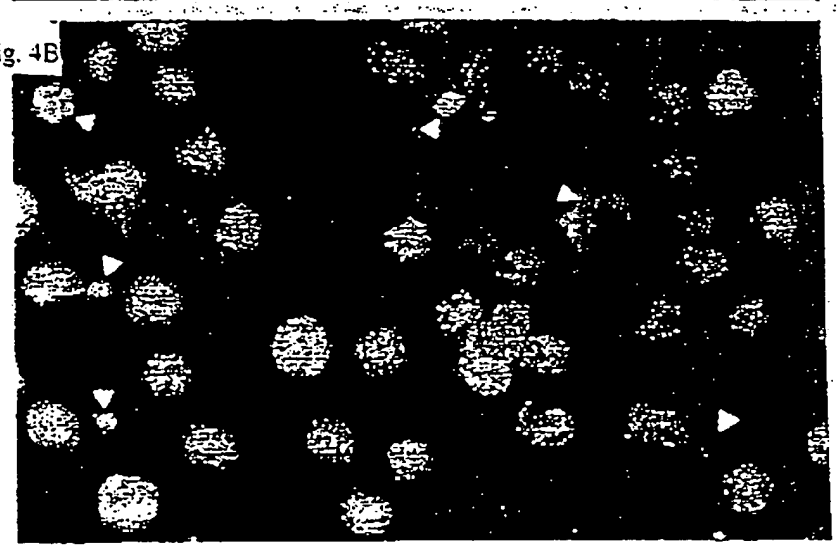


Fig. 4B



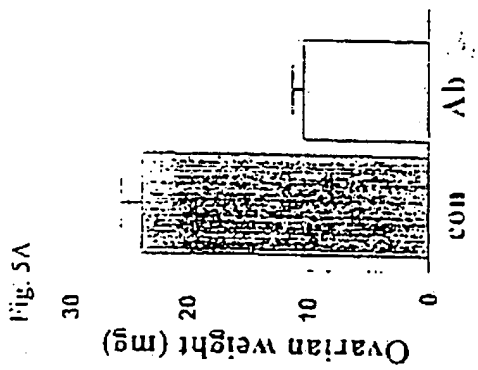


Fig. 5A

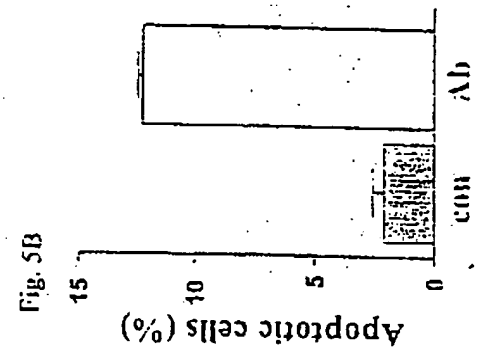


Fig. 5B

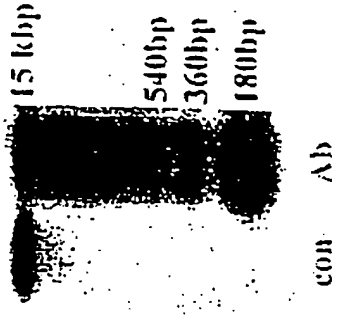


Fig. 5C

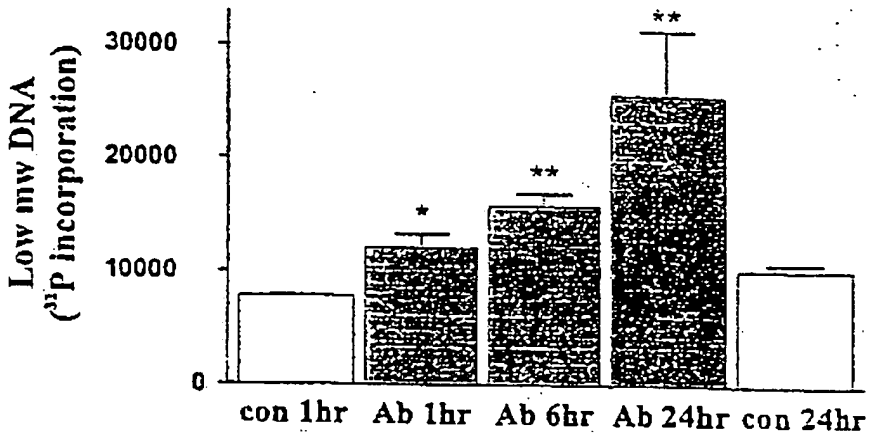

Low mw DNA
(^{32}P incorporation)
30000
20000
10000
0
*
**
**
con 1hr
Ab 1hr
Ab 6hr
Ab 24hr
con 24hr

Fig. 6

Co-localization of Apoptosis and Xiap & Hiap-2 on the rat ovary

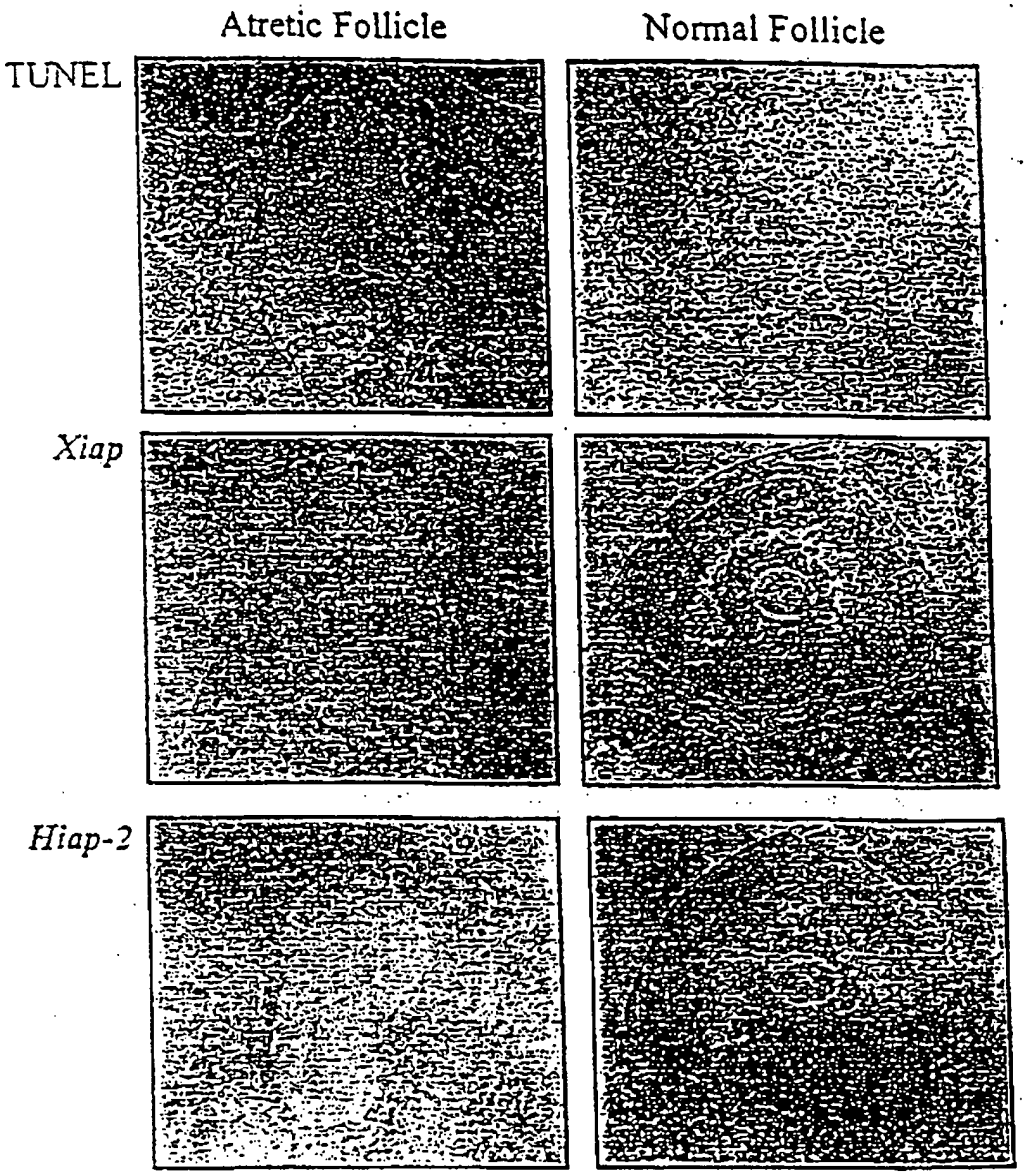
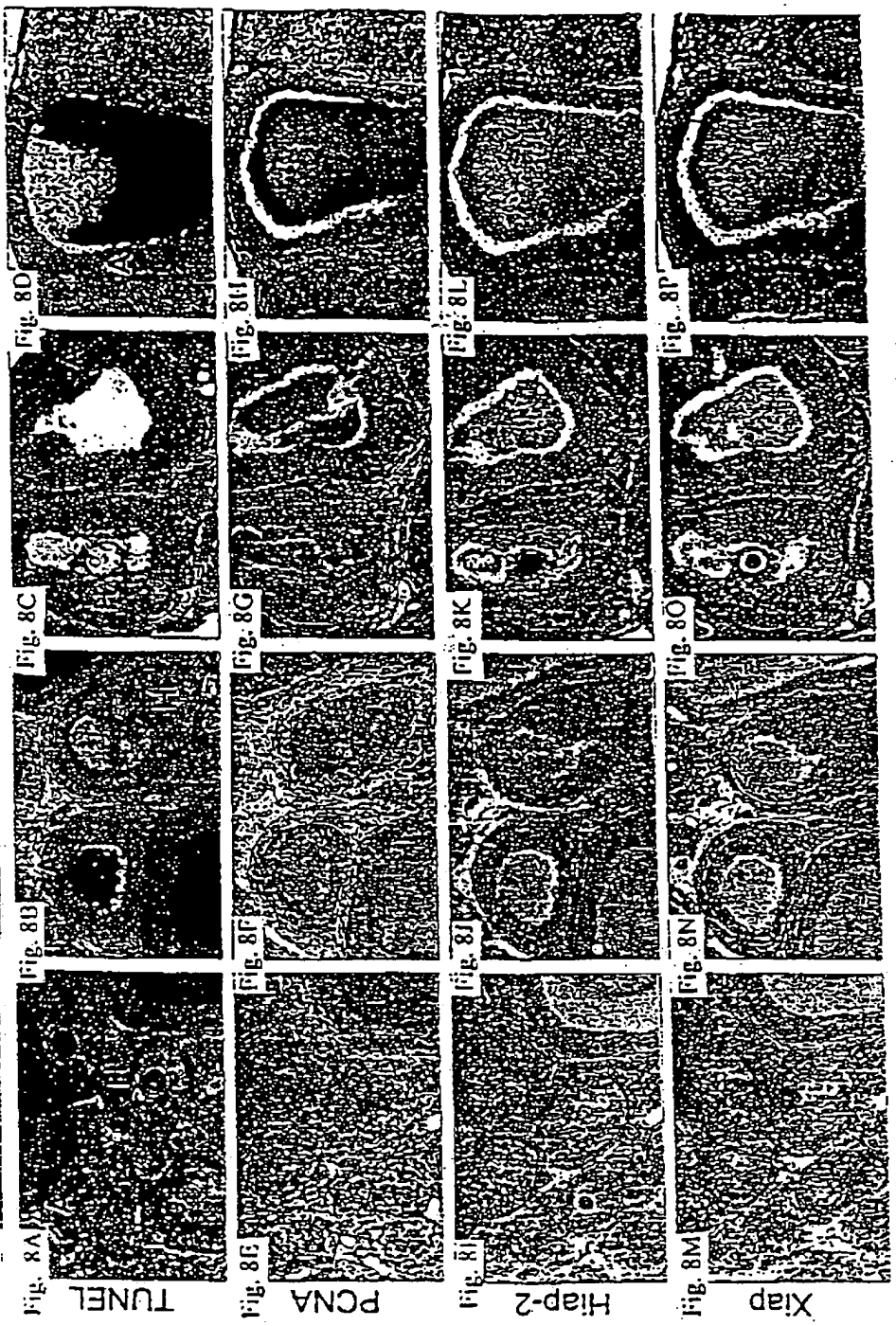
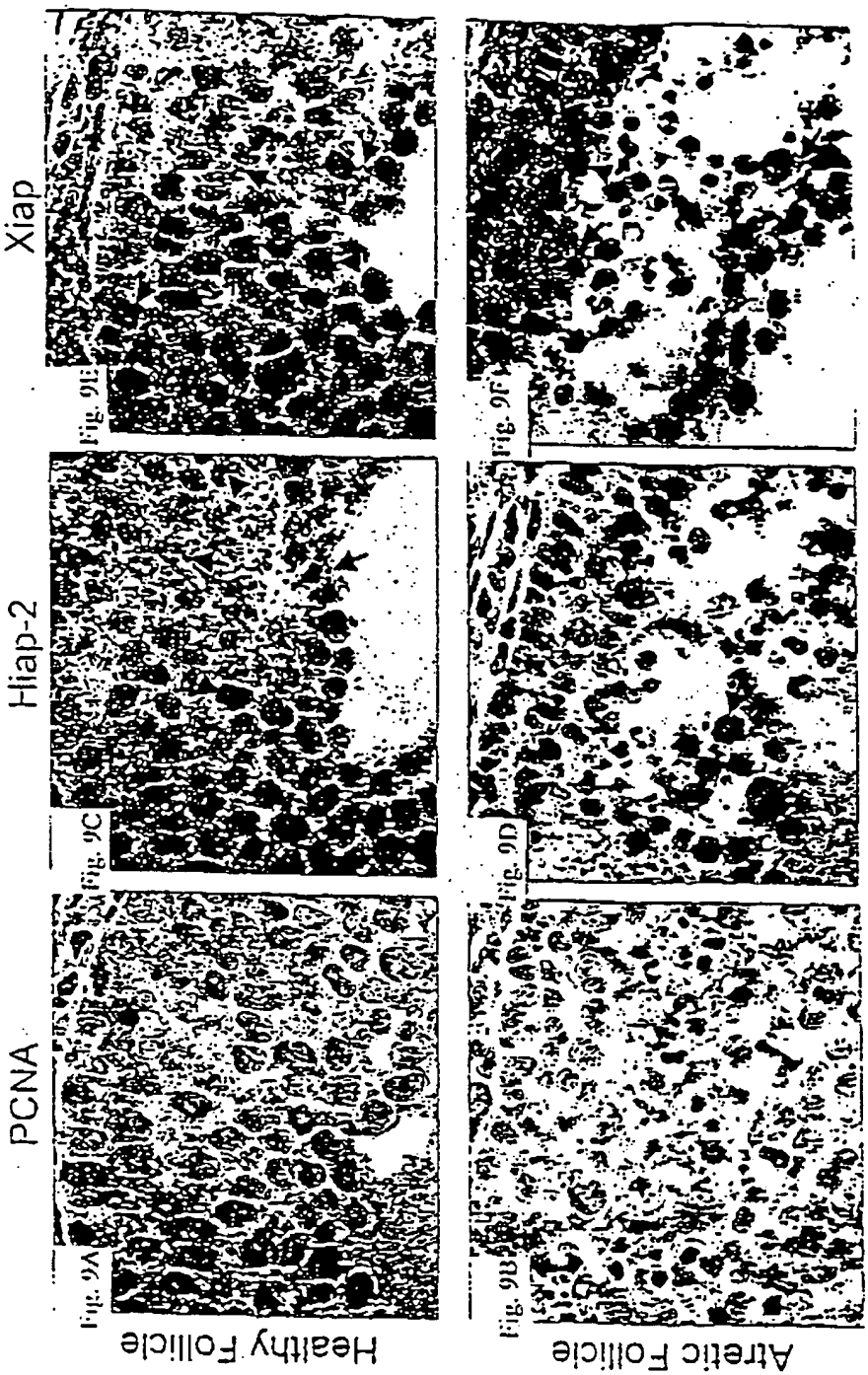


Fig. 7


Preantral Follicles
Early Antral Follicles
Antral Follicles
TUNEL
PCNA
Hiap-2
Xiap
Fig. 8A
Fig. 8B
Fig. 8C
Fig. 8D
Fig. 8E
Fig. 8F
Fig. 8G
Fig. 8H
Fig. 8I
Fig. 8J
Fig. 8K
Fig. 8L
Fig. 8M
Fig. 8N
Fig. 8O
Fig. 8P


PCNA
Hiap-2
Xiap
Healthy Follicle
Atretic Follicle
Fig. 9A
Fig. 9B
Fig. 9C
Fig. 9D
Fig. 9E
Fig. 9F

Follicular Stage-dependent Expression of Hiap-2 Protein in Rat Granulosa Cells

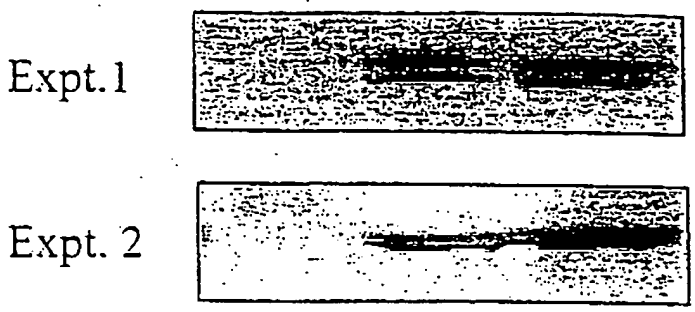
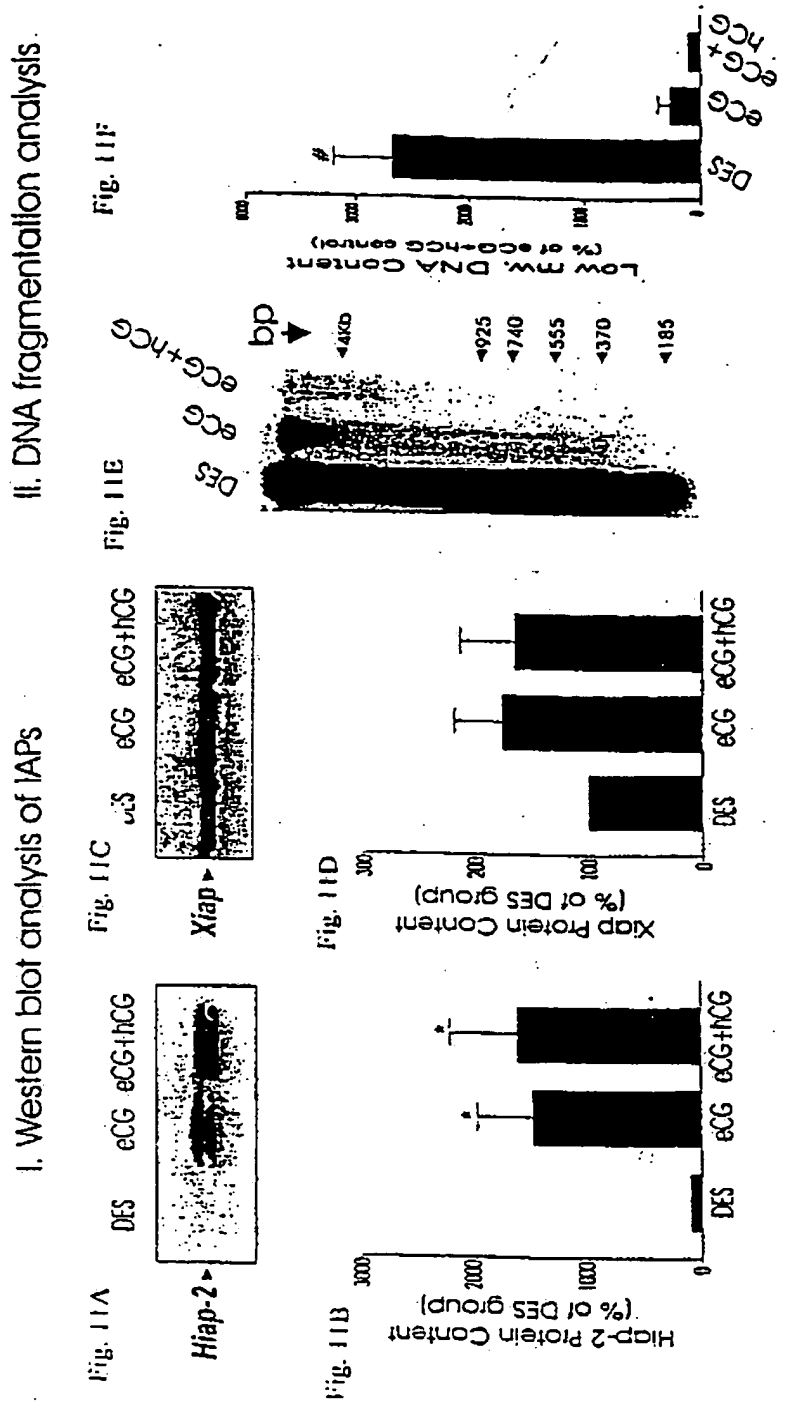
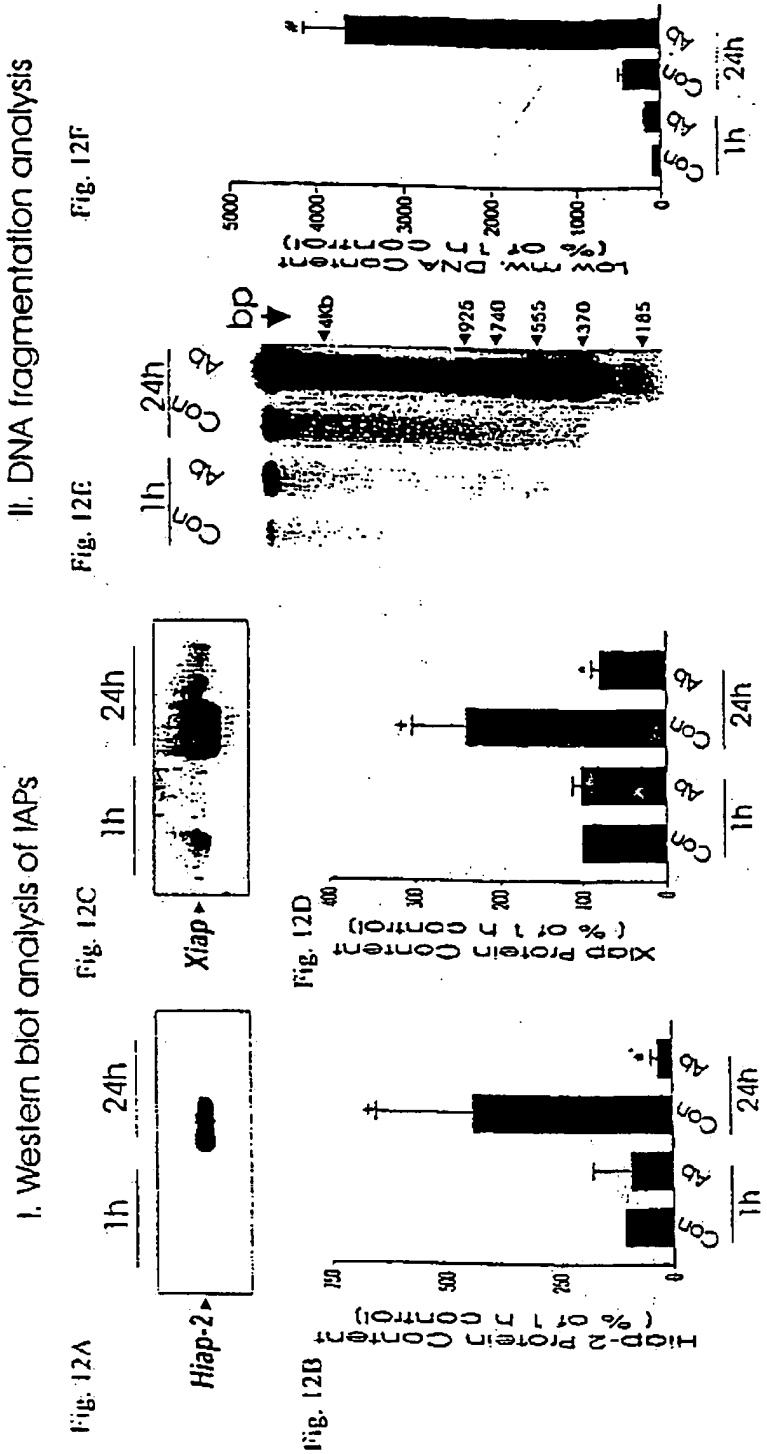


Fig. 10


I. Western blot analysis of IAPs
II. DNA fragmentation analysis
Fig. 11A
DES eCG eCG+hCG
Hiap-2
Fig. 11B
Hiap-2 Protein Content (% of DES group)
Fig. 11C
DES eCG eCG+hCG
Xiap
Fig. 11D
Xiap Protein Content (% of DES group)
Fig. 11E
DES eCG eCG+hCG
bp
4Kb
925
740
555
370
185
Fig. 11F
Low mw. DNA Content (% of eCG+hCG control)
DES eCG eCG+hCG


I. Western blot analysis of IAPs
II. DNA fragmentation analysis
Fig. 12A
Hiap-2
1h
24h
Fig. 12B
Hiap-2 Protein Content
(% of 1 h control)
Con
Ab
Fig. 12C
Xiap
Fig. 12D
Xiap Protein Content
(% of 1 h control)
Fig. 12E
bp
4kb
925
740
555
370
185
Con
Ab
Fig. 12F
Low mw DNA Content
(% of 1 h control)

Influence of Gonadotropin Withdrawal in vivo on Rat Granulosa cell Hiap-2 Protein Content

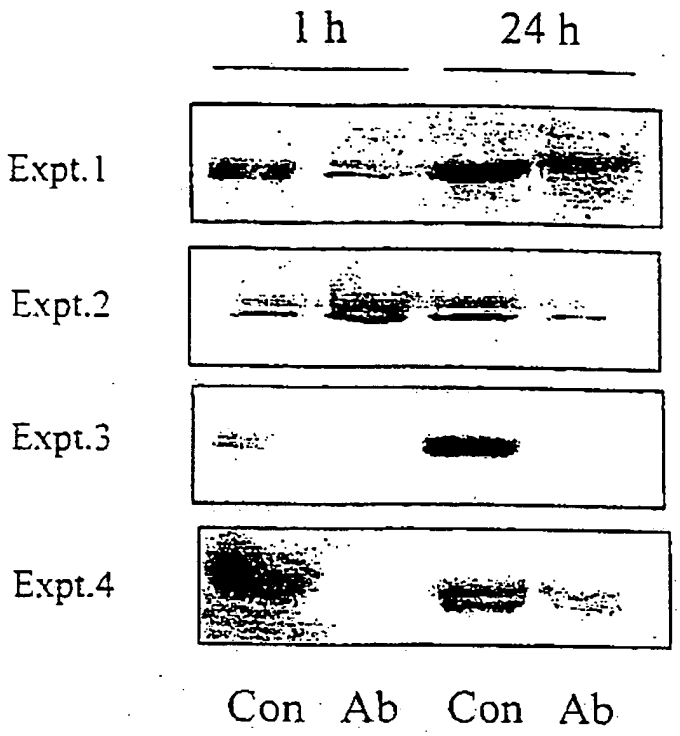


Fig. 13A

Influence of Gonadotropin Withdrawal in vivo on Rat Granulosa cell Xiap Protein Content

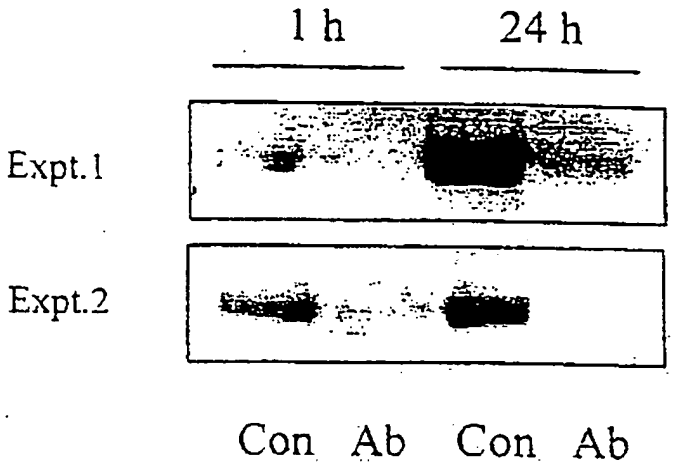


Fig. 13B

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(57) Abstract

The present invention demonstrates the ability of IAP member proteins and caspases which associate with them to modulate granulosa cell apoptosis, an event which leads to follicular atresia. This discovery allows the development and the identification of novel methods and compounds for controlling follicular atresia. Such methods and compounds are useful in the treatment of female infertility, and in the treatment and prevention of ovarian cancer.